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Review

The extrinsic polypeptides of Photosystem II

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1. Introduction

Photosystem II (PSII) is a pigment-protein complex found in the thylakoid membranes of plants, algae and cyanobacteria which drives light-induced electron transfer from water to plastoquinone with the concomitant production of molecular oxygen. Upon absorption of a photon by chlorophylls bound to other pigment-protein complexes known as the light harvesting complexes (LHCII), the excitation energy is ultimately transferred to the primary donor P_{680}, the structure of which is currently under debate (see Refs. [55,220] and references cited therein). The excitation of P_{680} leads to a transmembrane charge separation whereby an electron is transferred from P_{680} to a pheophytin molecule which is assumed to be bound by the D1 protein by analogy to the purple bacterial reaction centre. In order to stabilise the separated charges, the electron is further translocated to the primary quinone acceptor Q_A and from there to the secondary quinone acceptor Q_B which are both quinones located near the stromal side of PSII. After a second charge separation, Q_B becomes doubly reduced, protonated, and is then released into the thylakoid membrane. It is subsequently replaced by an oxidised quinone from the quinone pool (see Ref. [54] and references cited therein).

The oxidised primary electron donor P_{680}^+ is reduced by a redox-active tyrosine residue, Tyr_Z, which is located on the D1 protein. Tyr_Z^+ itself is reduced by a cluster of four manganese ions situated on the lumenal side of the membrane. In a series of four successive charge separations, four positive charge equivalents are accumulated at or close to the manganese cluster. After the fourth charge separation, two molecules of water become oxidised forming one molecule of oxygen (reviewed in Refs. [54,181,187,188]).

The structure and location of the manganese cluster are currently a field of intensive research. PSII is a large complex with many subunits, most of which are integral membrane proteins while others are peripherally associated with it [10,54,225]. The smallest PSII complex capable in water splitting consists of at least 8 polypeptides (see Fig. 1): the two reaction centre subunits D1 and D2 (which bind P_{680}, two pheophytins and probably the two quinones Q_A and Q_B), the cytochrome *b*-559 whose function is unknown, the chlorophyll *a* binding proteins CP47 and CP43, the extrinsic 33 kDa polypeptide, and at least one small membrane spanning protein (*Psb*I protein) with a molecular weight below 10 kDa [10,54,225]. Extensive mutational analysis of almost all of these subunits suggested the D1 protein as the major manganese binding protein (reviewed in Ref. [54]). The distance between Tyr_Z and the manganese cluster is currently under debate. Distances between 4.5 Å and greater than 15 Å have been suggested [12,72].

At the time of their discovery, the extrinsic polypeptides of PSII from higher plants, the so-called 33, 23, and 16 kDa polypeptides, were thought to be directly involved in water splitting since the removal of the 23 and the 16 kDa polypeptides led to a (partial) inactivation of oxygen evolution [4,117] and the removal of all three extrinsic polypeptides by alkaline Tris-washing [3,116,239] to a total loss of activity with concomitant release of manganese. In addition, there were reports of the isolation of the 33 kDa protein (and others) with bound manganese [1,243]. However, the release of the 23 and 16 kDa polypeptides was later found to be associated with a release of Ca^{2+} [40,70] and Cl^- [8,51,163] which turned out to be the sole cause of loss of activity. Moreover, methods were developed by which the 33 kDa polypeptide could be released without concomitant release of manganese [151,169]. These findings demonstrated that the extrinsic polypeptides do not provide the major binding site for the manganese cluster.

In this review the role of the extrinsic proteins in

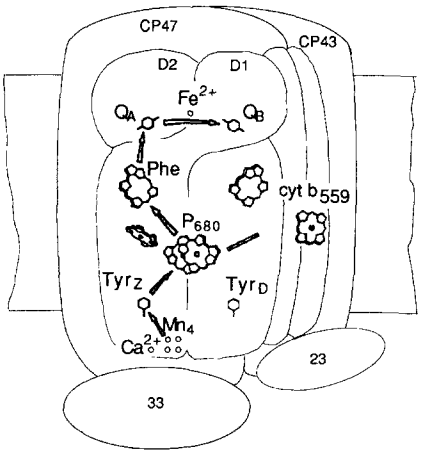

Fig. 1. A schematic model of Photosystem II.

oxygen evolution and their location will be discussed (Section 4, 5). In addition, a section on the biosynthesis and the protein import into the thylakoid lumen of these proteins has been included (Section 3), reflecting the recent improvement in the understanding of this area. The stoichiometry of the extrinsic polypeptides and an overview over their isolation is given in Section 2.

Several different nomenclatures are used in the literature for the extrinsic polypeptides. The three extrinsic polypeptides of higher plant Photosystem II are usually named according to the apparent molecular mass of the spinach proteins on SDS-gels. The apparent molecular masses of the polypeptides from higher plants are 33 kDa (sometimes 34 kDa), 23 (or 24) kDa and 16–18 kDa. Their true molecular masses have been determined by protein and/or DNA sequencing yielding values of 26.5, 20 and 17 kDa, respectively [99,168,219]. The genes for the extrinsic polypeptides have been named *psbO*, *psbP* and *psbQ*. Occasionally the corresponding proteins are referred to as PSII-O, PSII-P and PSII-Q. In *Chlamydomonas reinhardtii* the polypeptides are named oxygen-evolving enhancer (OEE) 1, 2 and 3 where OEE1 is the largest, and OEE3 is the smallest polypeptide. In cyanobacteria only the largest of the three polypeptides exists [209]. The gene for that protein has been termed *psbO*, *psb1* or *woxA* (for water-oxidising). The largest polypeptide is also sometimes referred to as manganese-stabilising protein. In this review the extrinsic polypeptides will be referred to as 33, 23 and 16 kDa polypeptides.

In cyanobacteria, although the 23 and 16 kDa polypeptides have not been found, one or two other extrinsic polypeptides exist on the lumenal side of PSII. These are the low-potential cytochrome *c*-550 and a polypeptide of 9–12 kDa. The latter was found until now only in thermophilic cyanobacteria. The gene for cytochrome *c*-550 has been termed *psbV* [205].

2. Stoichiometry and isolation

2.1. The stoichiometry of the extrinsic polypeptides

The stoichiometry of the 33, 23 and 16 kDa polypeptides was determined to be 1:1:1 [9,160]. However, different numbers of polypeptides on a per reaction centre basis have been reported. A ratio of one 33 kDa polypeptide per PSII reaction centre has been estimated by rocket immuno-electrophoresis, radial immuno-diffusion and densitometry of Coomassie blue-stained SDS-gels [9,160]. Scatchard blot analysis of reconstitution experiments of the 23 kDa polypeptide to NaCl-treated PSII resulted in a ratio of one 23 kDa polypeptide per PSII [155]. In a cross-linking experiment, the total disappearance of the 33 kDa protein and CP47 with concomitant appearance of a 80 kDa cross-linked product (33 kDa protein-CP47) was observed in SDS-gels and Western blots [59]. This was taken as evidence for a 1:1 stoichiometry of the two proteins. However, no evidence was presented that the cross-linked product contains the two proteins in a 1:1 ratio. Moreover, at the high concentration of cross-linker used, it is possible that proteins became modified so that they did not focus during SDS-gel electrophoresis.

Low resolution structures of PSII were obtained by several groups using electron microscopy of single PSII particles or of two-dimensional crystals. Recently, difference maps of intact and Tris-washed PSII were obtained [21,65,90,190], see also Ref. [79] and electron densities were assigned for the 33 kDa polypeptide [21] or all three extrinsic polypeptides [65,90]. The results obtained favoured a ratio of one extrinsic polypeptide per PSII. However, the assignment of the differences in electron density to the extrinsic polypeptide(s) are weakened by the fact that differences in electron density due to conformational changes in the intrinsic proteins were also observed.

A ratio of two 33 kDa polypeptides per PSII reaction centre was estimated by immuno-quantification of the 33 kDa polypeptide in Western blots [235]. Recently rebinding experiments were performed with PSII preparations lacking the 33 kDa protein which were proposed to differ in their manganese content (0, 2 and 4 manganese per PSII) [127]. In these reconstitution studies the data were fitted with two binding constants. However, this fitting might not be justified because of the scattering of the data. Another problem might be that the samples contain a heterogeneity in the manganese content per reaction centre. Even the most intact PSII preparations contain a fraction of reaction centres without functional manganese complex. This fraction might

be larger after the $CaCl_2$-treatment since the manganese complex is rather instable in the absence of the 33 kDa polypeptide. A heterogeneity in the manganese content might also exist in the sample which was supposed to contain an average of 0 and 2 Mn per PSII, which was not proven. Therefore, it cannot be excluded that the two binding constants found originate from two (or more) populations of PSII reaction centres which differ in their manganese content. If there are two binding sites for the 33 kDa polypeptide in PSII then they seem to be of very similar affinity.

The ratio of two 33 kDa polypeptides per PSII was supported by the analysis of the amount of unbound 33 kDa polypeptide in a similar reconstitution experiment. Betts et al. [18] found that in reconstitution studies where the 33 kDa polypeptide was incubated with urea/NaCl-treated PSII at molar ration of 2:1, 90% of the 33 kDa polypeptide was rebound, whereas only two-thirds of the 33 kDa polypeptide was bound when the ratio was 3:1. However, this is in clear contradiction with the findings of Miyao and Murata [155].

In summary, up to now most studies indicate that the ratio of 33 kDa polypeptide per reaction centre is 1:1, whereas the more recent studies indicate a ratio of 2:1. Assuming that there are two 33 kDa polypeptides per PSII reaction centre, both have (almost) the same affinity [127]. This makes it difficult to distinguish between the two binding sites. It is not clear whether both 33 kDa polypeptides are involved in oxygen evolution. One possibility to distinguish the two binding sites would be site-directed mutagenesis of amino acid residues of the 33 kDa polypeptide which might be involved in binding to PSII. This might lower the affinity of only one of the two 33 kDa polypeptides bound to PSII and could provide further evidence for a ratio of two 33 kDa polypeptides per PSII reaction centre.

2.2. *Isolation of the extrinsic polypeptides*

The extrinsic polypeptides are usually isolated by procedures which involve their release from PSII-enriched membranes and then their purification by anion exchange chromatography. Solutions of 1 M NaCl extract the 23 and 16 kDa polypeptides [4,117] along with some contaminants, including DNA [149]. The disadvantage of this procedure is that the polypeptides often become degraded, in the subsequent dialysis, by protease(s) released from the PSII membranes and activated by the NaCl treatment [118]. Two proteases involved in the degradation of the polypeptides have been characterised recently [121,122]. The polypeptides can be purified more easily when they are released by washing PSII with 1 M $CaCl_2$ [169] or 1 M Tris (pH 8.8) [239] or by butanol/water phase partitioning [241]. The subsequent dialysis of $CaCl_2$-extracts does not lead to any degradation. Either the protease(s) are not released or they are not activated by $CaCl_2$. Because of their quite different isoelectric points (pI = 5.1, 6.5 and 9.2 for the 33, 23 and 16 kDa polypeptides, respectively [114,242]), the three polypeptides can be quite easily separated by ion exchange chromatography [118,241] or by isoelectric focusing [242]. Since the 23 kDa polypeptide has some tendency to aggregate with itself or with the 33 kDa polypeptide at low ionic strength, it was found that a high salt concentration should be maintained throughout the isolation procedure.

A selective extraction procedure for the isolation of each polypeptide has been described [240]. The 16 kDa polypeptide can be selectively released by a treatment with 0.5 M NaCl in the presence of 20% methanol. Then, the 23 kDa polypeptide can be released by a subsequent treatment with 2 M NaCl and finally the 33 kDa polypeptide is removed using 0.8 M Tris (pH 8.4).

Higher yields of the extrinsic polypeptides were obtained when they were directly extracted from thylakoid membranes by treatment with cold acetone. However, this procedure requires more chromatographic steps for their purification since several proteins from protein complexes other than PSII were also extracted [9,100,115].

The cytochrome *c*-550, which has a redox midpoint potential of −260 mV [89] has been isolated from various cyanobacteria. Previously, this cytochrome was assumed to be involved in secondary electron transfer reactions in the cytoplasm, but recent investigations suggested a role in oxygen evolution [202–204]. Particularly clear evidence against a role in the secondary electron transfer is that the cloned genes for cytochrome *c*-550 were found to encode precursor proteins with N-terminal thylakoid

transfer peptides [165,205], indicating that the proteins are localised in the thylakoid lumen or in the periplasm. The binding strength of this cytochrome to PSII, and therefore the method for its release, varies significantly depending upon the species. The protein can be released from the thylakoid membrane or PSII by treatment with various salts [88,205] or detergents [86,113,165]. However, in some species it is already released during cell breakage [165].

3. Biogenesis and protein import

3.1. Protein expression

Photosystem II from higher plants and green algae consists of protein subunits which are both chloroplast and nuclear-encoded. The 33, 23 and 16 kDa extrinsic polypeptides of PSII are all nuclear-encoded [99,219] and their translation takes place at soluble ribosomes in the cytoplasm. Little is known about the regulation of the expression of these extrinsic polypeptides, in particular, the mechanism by which their expression is coupled to the expression of the chloroplast-encoded PSII subunits remains poorly understood.

The influence of light on the amount of mRNA and protein synthesised has been studied in various plants. The mRNA level of the 33 kDa polypeptide in etiolated spinach plants is only one-tenth of that in plants grown under white light, although no significant difference in the amount of accumulated protein has been observed [177,234]. Moreover, anti-sense RNA expression led to a decrease of the 23 and the 33 kDa polypeptide mRNA to lower then 10% and 5%, respectively, without any decrease in the protein level [177]. Etiolated barley plants contain approx. 50% of the 33 and 23 kDa polypeptide compared to plants grown under white light, whereas the 16 kDa polypeptide is only present in trace amounts [189]. However, no analysis of the mRNA levels were presented.

The dependence of the mRNA levels for the 23 kDa protein on the wavelength of light under which the seedlings were grown was also investigated [233]. The amount of mRNA formed was stimulated by far-red light, indicating phytochrome control of transcription. However, up to 25% of mRNA could also be observed in dark-grown plants compared to plants grown under red light. No data on the amount of protein present in the plant cells have been shown.

Overexpression of the 23 kDa polypeptide in tobacco [177] or of the 33 kDa polypeptide in *Chlamydomonas reinhardtii* [135] resulted in an increased mRNA and protein level. This indicates that there is no feedback regulation for the expression of these polypeptides.

In conclusion, an mRNA level lower than in plants grown under normal conditions results in an increase of translation but over-expression of the genes does not lead to reduced translation of the mRNAs. Except for the 16 kDa polypeptide from spinach, the effect of light on the protein level in the plants is not dramatic.

In contrast to etioplasts of higher plants, there is no 33 kDa polypeptide present in etioplasts of dark-grown *Euglena gracilis* Z [158]. When dark-grown *Euglena* cells were illuminated, the amount of the 33 kDa polypeptide increased in parallel with the development of oxygen-evolving activity. However, the appearance of electron transfer from Tyr_Z to Q_B developed much faster than oxygen evolution [158]. Therefore, the accumulation of the 33 kDa protein seemed to be the rate-limiting step in the assembly of PSII in this species. The presence of the extrinsic polypeptides in etioplasts of higher plants might allow the faster development of oxygen-evolving activity upon illumination. The minimisation of the length of time that each PSII must spend prior to its acquisition of oxygen evolution would be desirable considering the potential for photodamage in centres without a stable manganese complex. Due to a very different life cycle, *Euglena* might not need the fast adaptation to light.

3.2. Protein import

The three extrinsic polypeptides of PSII are translated on soluble ribosomes in the cytoplasm as precursor polypeptides carrying N-terminal transit peptides which consist of two domains. The first domain, the so-called chloroplast-targeting domain, directs the precursor polypeptides into the stroma of the chloroplasts where it is cleaved. This process will not be discussed here in detail since the extrinsic polypeptides represent only a minor fraction of the im-

ported proteins. Readers interested in this topic should consult Refs. [52,77,106] for recent reviews.

The second domain of the transit peptide, the so-called thylakoid transfer domain, directs the polypeptides into the thylakoid lumen where they are processed to their mature form. The transfer of the three extrinsic polypeptides across the thylakoid membrane shows some similarities to protein translocation in bacteria. Indeed, the thylakoid transfer domain of the transit peptides is similar to bacterial signal peptides which direct proteins into the periplasm (Fig. 2) [228]. The N-terminal part is variable in length, hydrophilic, and has a net positive charge; the central part is hydrophobic and its secondary structure is predicted to be α-helical; the C-terminal part usually contains alanine residues in position -3 and -1 (relative to the cleavage site of the signal peptidase) and constitutes the recognition site for the thylakoidal processing peptidase of higher plants and for the bacterial signal peptidase. In vitro experiments have shown that the substrate specificity of the *Escherichia coli* signal peptidase is, indeed, identical to the thylakoid processing peptidase, which processes the lumenal proteins to their mature form [7,81]. The functional equivalence of the thylakoid transfer domain of the 33 kDa polypeptide and of plastocyanin with bacterial signal peptides has been shown by expression of these polypeptides in *E. coli* and their export to the periplasm [80,194]. These similarities are in line with the endosymbiotic theory of the origin of chloroplasts, i.e., that free-living (ancestors of) cyanobacteria evolved into chloroplasts within the eukaryotic cell.

```
E.coli-lamB                    MMITLRKLPLAVAVAAGVMSAQAMA
E.coli-lamB19K                 MMITLRKLPLAVAVAAGVKSAQAMA

Spinach-PC                  ...KNVGAAVVATAAAGLLAGNAMA
Spinach-33 kDa              ...KLAGLALATSALI---ASGANA
Spinach-23 kDa              ...RRLALT-VLIGAAVGSKVSPADA
Spinach-16 kDa              ...RRAMLGFVAAGLASGSFVKAVLA
Pea-16 kDa                  ...RRAVIGLVATGIVGGALSQAARA
Barley-PsaN                 ...RRSALLGLAAVFAATAASAGSARA
Cotton-PsbT                 ...RREMM-FAAAAAAICSVAGVATA
Spinach-PPO              ...RRNILLGLGGMYAALGSEGANYYNTLA

Synechocystis-33 kDa          MRFRPSIVALLSVCFGLLTFLYSGSAFA
Synechococcus-33 kDa          MRYRAFLAAFLAVCLGVLTACSSGPTAA
Anabaena-33 kDa               MRYRALIVAILAVCLGLLTACSERSASS
S.elongatus-33 kDa            MKYRILMATLLAVCLGIFSL--SAPAFA
Synechocystis-PC            MSKKFLTILAGLLLVVSSFFLSVSPAAAA
Synechocystis-cyt c-553  MFKLFNQASRIFFGIALPCLIFLGGIFSLGNTALA
Synechococcus-cyt c-553       MKRIL-GTAIAALVVLL-AFIA--PAQA
Anabaena-cyt c-553            MKKIFSLVLLG-IALF--TFAFSSPALA
Synechocystis-cyt c-550       MKR-FFLVAIASVLFFFNTM--VGSANA
Synechocystis-PsaF            MKH--LLALLLAFTLWFNF---APSASA

Synechococcus-RhdA  MSVRSLRWPRQKAFLAVISLVVAVLLAVPGWLTPATA
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Fig. 2. Sequence comparison of signal peptides and thylakoid transfer peptides: *E. coli* LamB [208]; plastocyanin (PC) [185]; 33 kDa polypeptide [219]; spinach 23 and 16 kDa polypeptide [99]; maize 16 kDa polypeptide [64]; PsaN [108]; Cotton PsbT [103]; Spinach PPO [85]; cyanobacterial 33 kDa polypeptides (see Fig. 3); *Synechocystis* PCC 6803 plastocyanin (PC) [33]; *Synechocystis* PCC 6803 cyt *c*-553 [247]; *Synechococcus* PCC 7942 cyt *c*-553 [126]; *Anabaena* PCC 7937 cyt *c*-553 [28]; *Synechocystis* PCC 6803 cyt *c*-550 [205]; *Synechocystis* PCC 6803 PsaF [43]; *Synechococcus* PCC 7942 *rhdA* [125]. (In order to keep the sequence alignment as clear as possible, not all plant transit sequences are shown but only those which differ in the position of the positively charged amino acid residue close to the cleavage site. A more complete alignment can be found in Ref. [48].)

It has been shown recently that there are four different mechanisms of protein translocation across the thylakoid membrane. The first mechanism is related to bacterial protein translocation where proteins are translocated into the periplasm (Sec-related mechanism). The second translocation mechanism involves only a proton gradient over the thylakoid membrane but does not depend on ATP. CF_0II, a subunit of the ATP synthase, is inserted into the thylakoid membrane by a third mechanism which does not require a ΔpH, ATP or any of the 'stromal factors' [144]. Finally, the LHCII apo-protein is incorporated by a mechanism dependent on 'stromal factors' and requires ATP as well as a ΔpH [47].

The 33 kDa polypeptide belongs to a class of proteins in which they are translocated by the first mechanism, whereas the 23 and the 16 kDa polypeptide belongs to the class of proteins which is translocated by the second mechanism [47,48,93,107]. The translocation of the 33 kDa polypeptide is absolutely ATP-dependent and facilitated by a proton gradient over the thylakoid membrane, whereas the translocation of the 23 and 16 kDa proteins requires only the proton gradient (reviewed in Ref. [183]). Secondly, import of the 33 kDa polypeptide requires 'stromal factor(s)'.

Recent work has apparently clarified the nature of the so-called 'stromal factor(s)' involved in the translocation of the 33 kDa polypeptide and plastocyanin. It was found that azide, an inhibitor of the bacterial SecA protein involved in protein export,

inhibits the thylakoid transfer of the 33 kDa polypeptide and plastocyanin, whereas the import of the 23 and 16 kDa polypeptide was not affected [48,84,109]. Very recently, a SecA protein was isolated from chloroplasts [245] and it was shown that this protein is, indeed, the 'stromal factor' required for the import of the 33 kDa polypeptide and plastocyanin [166,245]. Antibodies raised against this protein inhibit the import of the 33 kDa polypeptide into the thylakoid lumen but not that of the 23 kDa polypeptide [161]. Depletion of SecA from stromal extracts led to an inhibition of the import of the 33 kDa protein [245]. In addition, the cDNA for the SecA protein from pea and spinach chloroplasts [15,161] as well as the gene for the SecY protein from *Arabidopsis thaliana* chloroplasts [124] have been cloned. However, the existence and/or the involvement in a certain mechanism of additional homologues of proteins which participate in bacterial protein export remains to be established. These might include SecB and/or a signal recognition particle homologue. The involvement of a chloroplast homologue to the SRP54, a subunit of the cytosolic signal recognition particle from mammalian cells involved in protein translocation into the lumen of the endoplasmic reticulum, has been shown for the integration of the apo-protein of LHCII [128].

A detailed characterisation of the kinetics and specificities of the import pathways of proteins into and across the thylakoid membrane was carried out by Cline and co-workers [48]. They showed that thylakoid translocation of the 33 and the 23 kDa polypeptide is saturable, indicating that the protein translocation involve enzymatic reaction(s) not only for the 33 but also for the 23 kDa polypeptide. Moreover, they showed by competition experiments that the 23 and the 16 kDa polypeptides, but not the 33 kDa polypeptide, compete with each other for the same protein translocation system. The 33 kDa polypeptide competed with plastocyanin for import into the thylakoid lumen. Interestingly, none of the identified proteins which can be imported independent of ATP (i.e., 23 and 16 kDa polypeptides, PsaN, PsbT [84,182]) is present in cyanobacteria [183]. It has been suggested, therefore, that they are imported into the thylakoid lumen by a more recently developed pathway, whereas the 33 kDa polypeptide, plastocyanin and the lumenal Photosystem I subunit PsaF, which are all present in both cyanobacteria and plants, are imported by a more ancient bacterial-type pathway, which is related to the Sec-machinery (reviewed in Ref. [183]).

Recently it was found that, in the stroma, the 23 kDa polypeptide precursor does not form a stable interaction with chaperonins or other proteins. Moreover, it seems to have a rather tightly folded structure since it shows the same protease sensitivity as the mature protein [50]. This is in contrast to bacterial precursors of periplasmic proteins which interact with chaperonins and which are thought to have a rather loose structure even in the isolated form.

At present, it is not totally clear which are the signals that determine whether a protein is translocated over the thylakoid membrane by one pathway or the other. Experiments in which lumenal proteins were translocated either by their own thylakoid transfer peptide or by those of other lumenal proteins showed that the mechanism of import is determined by the transfer peptide and not by the mature protein [46,84,182]. The thylakoid transfer peptide of proteins which are translocated by the ΔpH-dependent pathway contain a doublet of arginine residues directly adjoining the hydrophobic domain on the N-terminal side, whereas the proteins translocated by the Sec-related pathway have only a single lysine residue at the corresponding position (Fig. 2). In addition, despite the similarities with bacterial signal peptides mentioned above, the thylakoid transfer domains of the transit peptides of the 23 and 16 kDa polypeptides have a feature which is not present in bacterial signal peptides. In almost all organisms sequenced so far, the transit peptides of the 23 and 16 kDa polypeptides have a positive charged amino acid residue near the cleavage site (Fig. 2) (reviewed in Ref. [112]). In the 23 kDa polypeptide, the positive charged residue is at position -7 (relative to the cleavage site of the thylakoid processing peptidase), while in the 16 kDa polypeptide it occurs at position -5 or -2. It has been shown for the *E. coli* protein LamB that a positively charged residue at position -7 inhibits the export of the protein [208]. Consistent with this finding is that the 16 kDa polypeptide could not be exported into the periplasm of *E. coli* by its own thylakoid transfer peptide ([123], T. Kuwabara, personal communication). The fact that the 23 kDa protein, fused to an *E. coli* signal pep-

tide, could be exported to the periplasm [192] confirmed that this is not an effect of the mature protein but of the signal peptide. It is probably important that the precursors of the lumenal proteins are well separated between the two types of import pathways for the thylakoid lumen since, otherwise, the bacterial-type pathway might become blocked by the proteins translocated by the ΔpH-dependent pathway.

Replacement of only one of the two arginine residues in the thylakoid transfer peptide by lysine results in a strong inhibition or even a total block of the protein import, indicating that the arginine doublet is a recognition signal for the translocation machinery [41]. However, the protein with the arginine-to-lysine mutation is also not translocated by the Sec-related pathway. Moreover, introduction of an arginine doublet into the thylakoid transfer domain of the plastocyanin transit peptide did not lead to a translocation of the protein via the ΔpH dependent pathway but to a slight inhibition of the translocation via the Sec-related machinery. This indicates that there are additional factors which influence the choice of the pathway. These additional factors seem not to be located in the hydrophobic core of the thylakoid transfer domain since replacement of the hydrophobic core in the transit peptide of the 16 kDa polypeptide by part of a putative transmembrane helix of PsbS, a subunit of PSII presumed to be involved in binding of chlorophyll *a* and *b* [67,232], had no influence on the choice of translocation pathway [41]. However, the translocation pathway of PsbS itself has not been studied yet. PsbS is supposed to be a member of the Cab protein family. Another member of this family was shown to be imported by an ATP-dependent pathway [47].

Another targeting signal might be the positively charged residue close to the cleavage site of the thylakoid processing peptidase. As mentioned above, such a residue was shown to inhibit export of LamB in *E. coli*. Therefore, it might be interesting to see whether an arginine doublet before and a lysine residue after the hydrophobic domain in the transit peptide of plastocyanin leads to a translocation over the thylakoid membrane via the Sec-related pathway.

It should be pointed out that there are a few exceptions to the general pattern of the distribution of positively charged residues in the thylakoid transfer domain of the transit peptide. Although cytochrome *f* is an 'old' protein present in cyanobacteria, the pre-peptide contains an arginine residue in position −7 relative to the cleavage site [6]. However, it is chloroplast-encoded and membrane integration probably occurs cotranslationally. Therefore, it might be that the mechanism of protein translocation/insertion is somehow different from that of the extrinsic polypeptides of PSII. However, recently a mutant of *C. reinhardtii* was isolated which interfered with the protein translocation of the 33 kDa polypeptide, plastocyanin, PsaF and cytochrome *f* across the thylakoid membrane but not with the translocation of the 23 and 16 kDa polypeptides [227], indicating that cytochrome *f* might be translocated by the same mechanism as the 33 kDa polypeptide. Other exceptions are the PSII-T protein from cotton [103], the 23 kDa polypeptide from *C. reinhardtii* [137], and polyphenol oxidase [85] all of which do not contain a positive charged amino acid residue in the thylakoid transfer domain near the cleavage site of the thylakoid processing peptidase. The import of PSII-T is special in that there are three instead of two processing steps involved in import into the thylakoid lumen [103]. The thylakoid membrane transfer of polyphenol oxidase and the 23 kDa polypeptide from *C. reinhardtii* has not yet been studied.

Little is known about the translocation of proteins across the thylakoid membrane of cyanobacteria. Their thylakoid transfer peptides closely resemble bacterial signal peptides (Fig. 2). The only periplasmic localised protein from cyanobacteria which has been cloned and the signal sequence of which is therefore known is the rhodanese-like protein from *Synechococcus* PCC 7942 [125]. The N-terminus of its signal peptide contains more positively charged amino acid residues than the thylakoid targeting peptides (Fig. 2). Whether this is significant is not yet clear. The thylakoid transfer peptides of the 33 kDa polypeptide from *Synechococcus* PCC 7942 [119] and *Anabaena* PCC 7120 [22] have been shown to act as signal peptides in *E. coli*. (The function of the latter as a signal peptide in *E. coli* might appear somewhat surprising since it contains a positive charged residue at position −5. However, since the yield of protein expression is extremely low in this case, it might be that the majority of the recombinant protein is degraded before or during export.) It is still not clear whether the cyanobacteria have two differ-

ent transport systems for each cell compartment or whether the same transport system is able to transport proteins into both compartments. Two attempts were made to answer the question whether different transport systems exist for both compartments. In the first, cytoplasmic and thylakoid membranes were isolated from the cyanobacterium *Synechococcus* PCC 7492 and the SecY protein was detected by Western blotting in both membranes [162]. However, no Western blots with antibodies against marker proteins for the cytoplasmic and thylakoid membranes were carried out in order to prove that the isolated thylakoid membrane was not contaminated by the cytoplasmic membrane. In the second attempt a reporter gene was fused to the signal peptides of lumenal and periplasmic proteins. These chimeric proteins were expressed in the cyanobacterium *Synechococcus* PCC 7492 and the reporter protein was localised [133]. The results did not provide a clear answer, probably because some of the chimeric proteins expressed were not soluble under the experimental conditions and were co-isolated with the envelope. Therefore, the question whether there are one or more translocation machineries in cyanobacteria remains to be answered.

4. The function of the extrinsic polypeptides

4.1. The function of the 23 kDa and 16 kDa polypeptides

4.1.1. Dissociation and reconstitution experiments

The involvement of the 23 kDa and 16 kDa polypeptides in oxygen evolution was discovered by dissociation and reconstitution experiments using inside-out vesicles and PSII-enriched membranes. As mentioned in Section 2, treatment with 1 M NaCl releases these two proteins [4,117]. Subsequent reconstitution of these two polypeptides [4,150] or of the 23 kDa polypeptide alone [129], restored (partial) activity. The degree of loss and restoration of the activity was found to be extremely dependent on the experimental conditions. Further investigations revealed that addition of Ca^{2+} [68,152] and Cl^- [8,51,163] to the assay buffer restores activity without reconstitution of the extrinsic polypeptides. Reconstitution of the polypeptides in Ca^{2+}-free medium did not restore any activity at all [70].

The 23 kDa polypeptide either modulates the binding affinity or the on- and off-rate of binding of the Ca^{2+} ion. An influence of the 23 kDa polypeptide on the binding kinetics of Ca^{2+} was observed [70]. Recent data indicate that the Ca^{2+}-binding affinity is almost identical in the presence and absence of this polypeptide [2]. Therefore, the 23 kDa polypeptide influences only the kinetics of Ca^{2+} binding and release.

It was also observed that the Cl^- requirement for maximal oxygen evolution was modified by the 23 and 16 kDa polypeptides [5,8,51,91,152,163]. In the absence of the 23 kDa polypeptide, at least 30 mM Cl^- is necessary for maximal activity. Rebinding of the 23 kDa polypeptide reduces the Cl^- requirement for maximal oxygen evolution to 2–10 mM [5,91,155]. Additional rebinding of the 16 kDa polypeptide increases oxygen evolution slightly at Cl^--concentrations below 2–3 mM. Interestingly, these two extrinsic polypeptides seem not only to increase the binding affinity of Cl^- at PSII but Cl^- also seems to enhance the binding of the 23 kDa polypeptide [91].

There is a high variability in the reported extent of inhibition of oxygen evolution after NaCl-washing in the literature. Miyao and Murata showed that the degree of inhibition was dependent on whether the NaCl-treatment was carried out in the dark or in the light [154]. Boussac and Rutherford demonstrated that, indeed, the binding of Ca^{2+} depends on the S-state [23]. After release of the 23 and 16 kDa proteins by 1 M NaCl, Ca^{2+} is released during illumination, probably in the S_3-state since it is in this state where Ca^{2+} is most easily lost. The release of the 23 and 16 kDa polypeptides does not change the EPR properties of PSII ([24], earlier contradictory results are discussed therein). Release of Ca^{2+} has no effect on the EPR signals of the S_2-state [24] whereas in the formal S_3-state a split signal at $g = 2$ appeared which has been attributed to an organic radical interacting magnetically with the manganese cluster [26,27]. This signal is broader and PSII is more stable in the presence of the 23 and 16 kDa polypeptides [25]. Metal chelators have no effect on oxygen evolution in intact PSII-enriched membranes [70]. However, treatment with 1 M NaCl in the presence of EGTA or EDTA in the light releases not only Ca^{2+} but also modifies the manganese cluster as seen by its EPR signal [26,175]. A similar effect was observed

after treatment with citrate at low pH [25]. During this low pH treatment, the 23 and 16 kDa polypeptides are released [201] concomitantly with Ca^{2+} [174]. Upon adjusting the pH to 6.5, the polypeptides rebind to PSII, whereas Ca^{2+} rebinding is inhibited due to its chelation by citrate. The additional effect of this chelator has been suggested to originate from a binding of the chelator (citrate, EGTA, EDTA etc.) directly to the manganese cluster [25,222,246]. Modification of the structure of the manganese cluster by citrate treatment has been demonstrated by EXAFS [237].

In thermoluminescence studies, the 23 kDa polypeptide was reported to influence the stability of the $S_2Q_A^-$ state in Ca^{2+}-depleted PSII [92,176]. The different stability of this state was suggested to be due to a conformational change of the manganese cluster [92,176] or by direct binding of the 23 kDa protein to manganese in this PSII preparation [92]. However, the magnitude of the temperature shift of the thermoluminescence signal, due to the presence or absence of the 23 kDa polypeptide, differs greatly in the two reports. Moreover, the observed recombination reaction has been suggested to be $Tyr_D^+Q_A^-$ rather than $S_2Q_A^-$ [101].

In the absence of the 23 kDa polypeptide the manganese cluster becomes more susceptible to chemical attack. Bulky reductants which have no effect on the manganese cluster in intact PSII are able to over-reduce the manganese cluster resulting in the subsequent release of Mn^{2+} into the medium [71,216]. It was found that the Mn^{2+} formed by reductants can be trapped in a kind of aqueous cavity inside of PSII when the treatment is done in the presence of mM concentrations of Ca^{2+} to NaCl-washed PSII [141]. These Mn^{2+} ions can be re-oxidised by illumination and the activity recovered.

4.1.2. Deletion mutagenesis

Two *C. reinhardtii* mutants, BF25 and FUD39, have been isolated which are unable to synthesise the 23 kDa polypeptide [14,53,137]. Mutant BF25 showed only 5% of the wild-type oxygen-evolving activity and a low level of variable fluorescence. The number of PSII reaction centres was found to be comparable to wild-type in Ref. [137]. However, in Ref. [53] it was reported that the level of PSII present in the mutants was only 65% in BF25 and 30% in FUD39 compared to the wild-type. When grown under dim light, FUD39 showed nearly normal PSII levels, indicating that the 23 kDa polypeptide-less mutant has a greater susceptibility for photoinhibition [186]. In isolated thylakoids this effect was partially reversed by adding mM concentrations of Cl^-. However, no absolute values of oxygen evolution nor information about the Ca^{2+}-requirements for activity were reported which makes it difficult to judge whether the protecting effect of Cl^- is significant.

In conclusion, the role of the 23 kDa polypeptide is (1) to prevent Ca^{2+} from being released from PSII during the S-state turnover, (2) to create a high affinity Cl^--binding site, (3) to protect the manganese cluster from chemical attack, and (4) to stabilise the manganese cluster. The 16 kDa polypeptide seems also be involved in (2)–(4), although to a lower extent.

4.2. The function of the 33 kDa polypeptide

4.2.1. Dissociation and reconstitution experiments

Treatment of PSII-enriched membranes with 1 M Tris (pH 8.0) [239], alkaline pH [116] and 2.6 M urea [153] releases the 33, 23 and 16 kDa polypeptides along with some manganese ions of the cluster. Since 1 M NaCl releases only the 23 and 16 kDa polypeptides (see above) without releasing the manganese, the simultaneous loss of the 33 kDa polypeptide and the manganese cluster as well as reports of manganese being associated with the 33 kDa polypeptide under some conditions [1,243] were used as arguments to support a direct involvement of this polypeptide in manganese binding (reviewed in Ref. [13]). However, using treatments with 2.6 M urea/200 mM NaCl [151,153] or 1 M $CaCl_2$ [169,170] in the dark, the 33 kDa polypeptide could be released leaving the manganese cluster intact. In the absence of the 33 kDa polypeptide, however, the manganese cluster is rather unstable. Cl^- concentrations of higher than 100 mM are required to prevent the release of manganese [153,159] and in oxygen evolution assays under saturating light, the activity declines rather rapidly. The activity after the release of the 33 kDa polypeptide in the presence of 5 mM Ca^{2+} and 200 mM Cl^- is usually between 16 and 45% of the activity of intact PSII. The variation might be due to a different number of inactivated

reaction centres due to release of functional manganese and due to different amounts of reaction centres still containing the 33 kDa polypeptide [30]. Rebinding of the 33 kDa polypeptide restored the activity [151,153,170]. In the absence of the 33 kDa polypeptide the manganese cluster is more accessible from the outside as shown by the effects of chemicals such as reductants [213,216] or EDC (A.S., submitted).

There have been some reports that the S_2 multiline EPR signal could not be observed in PSII depleted of the 33 kDa polypeptide. It seems likely that this was due to the lack of sufficient Cl^- in the buffer since in the presence of 200 mM Cl^- the S_2 multiline EPR signal was indeed observed, although it was somewhat weaker than in intact PSII [136,210]. (Thermoluminescence data on PSII-enriched membranes lacking the 33 kDa polypeptide probably reflect the situation in which PSII is inhibited due to an insufficient amount of Cl^- and Ca^{2+}; [172,224]). The reason for the lack of multiline signal at low Cl^- concentration might be an over-reduction of the manganese cluster, since it has been observed that at low Cl^- concentration, manganese is slowly released from PSII [153]. It has been reported that in the absence of the 33 kDa polypeptide, Ca^{2+} was required for the observation of the multiline EPR signal [145]. This is rather surprising since the presence or absence of Ca^{2+} has no influence on the stability of the manganese cluster. However, in a recent study the multiline EPR signal could be detected in the absence of the 33 kDa polypeptide, although the ability of PSII lacking the 33 kDa polypeptide to form an EPR-detectable S_2-state is rather easily lost [197].

Flash-induced oxygen evolution measurements of PSII depleted of the extrinsic polypeptides in the presence of 10 mM Ca^{2+} and 200 mM Cl^- revealed a slightly [156] or strongly [173] damped period-four oscillation. Variation of the spacing in between the flashes revealed that specifically the S_3–S_0 transition was slower as compared to intact PSII. In the absence of the 33 kDa polypeptide the S_2-state and, to a lesser extent, the S_3-state are more stable [156] [1].

[1] Similar studies were carried out by others. However, the interpretation of their data is difficult since measurements were carried out at low Cl^- concentrations and in the absence of Ca^{2+}.

Up to this point, only the influence of the 33 kDa polypeptide on the properties of the manganese cluster have been discussed. The influence of the manganese cluster on binding of the 33 kDa polypeptide has also been studied. Measurements of the affinity of the 33 kDa protein to PSII revealed a K_d of 12 nM in the presence and 88 nM in the absence of the manganese complex [155]. Slightly lower values were obtained in a similar study [127]. In a different experiment it was shown that the 33 kDa polypeptide could be partially removed by 2 M NaCl in the absence of the manganese cluster [105], suggesting a weaker binding of the protein in the absence of the manganese complex.

The 33 kDa polypeptide from spinach and from *Synechococcus vulcanus* has been shown to be exchangeable to some extent. The spinach polypeptide could be bound to $CaCl_2$-washed PSII from *S. vulcanus* and vice versa. Partial restoration of the activity was observed [111]. It was reported that the 33 kDa polypeptide from *Arabidopsis thaliana* over-expressed in *E. coli* binds with a lower affinity to PSII from spinach than the spinach protein itself [17]. However, it was discovered later that the lower binding affinity was due to a partial misfolding of the recombinant polypeptide. It is now clear that the binding affinity of the 33 kDa polypeptide from both species is identical [18]. This is in agreement with the high sequence homology observed among the polypeptide from different species (see Fig. 3).

4.2.2. Deletion and site-directed mutagenesis

A mutant of *C. reinhardtii* has been characterised which was not able to synthesise the 33 kDa polypeptide due to an insertion in the gene [136]. In addition, the mutant contained only 10% of PSII compared to the wild-type. This was taken as evidence that the 33 kDa polypeptide was not only essential for oxygen evolution but also for PSII stability. A different interpretation would be that PSII in this mutant is more sensitive to photoinhibition and is therefore less stable [186]. In contrast to these findings deletion of the *psbO* gene encoding the 33 kDa protein in *Synechocystis* PCC 6803 [35,63,134,180] or *Synechococcus* PCC 7942 [20] did not lead to a loss of the ability to grow photoautotrophically. The number of PSII reaction centres was determined to be 80–95% of that found in the wild-type strain [36,180]. The

oxygen-evolving activity in intact cells was found to be 70% of the rate of the wild-type [35,134,180] and 33%–40% in the presence of an artificial electron acceptor [35,38].

The difference between *C. reinhardtii* and the cyanobacterial mutants lacking the 33 kDa polypeptide might be due to differences in the subunit composition on the lumenal side of PSII. In higher plants and *C. reinhardtii* there are the 33, 23 and 16 kDa polypeptides. In the absence of the 33 kDa polypeptide the binding affinity of the other two polypeptides is strongly reduced (see Section 5.2). In contrast, cyanobacteria lack the 23 and 16 kDa polypeptides. Instead, they have a low-potential cytochrome *c*-550 and, at least in the thermophilic strains, a 9–12 kDa polypeptide bound to the lumenal side of PSII. These two proteins can bind even in the absence of the 33 kDa protein (see Section 5.3). Therefore, in the *C. reinhardtii* mutant which is unable to synthesise the 33 kDa polypeptide, PSII is in fact lacking three and not only one polypeptide, whereas in cyanobacteria the cytochrome *c*-550 seems to be bound to PSII even in the absence of the 33 kDa polypeptide.

In wild-type cells of *Synechocystis* PCC 6803, the absence of Ca^{2+} and Cl^- had little [180] or a moderate [63] effect on the photosynthetic growth of the cells. However, absence of Ca^{2+} from the growth medium of the *psbO*$^-$ strain prevented any cell growth [63,180]. Possible reasons for this phenomenon are discussed in chapter 4.2.3.

The effect of Cl^- on the growth rate of the *psbO*$^-$-strain is controversial. Whereas Philbrick et al. [180] found no difference compared to the wild-type strain, Engels et al. [63] reported that their deletion strain could not grow without Cl^-. They suggested that the difference might originate from differences in the growth conditions. The Cl^- concentration in the cells might under some circumstances be high enough to support photosynthetic growth for some time. No effect of the absence of Ca^{2+} or Cl^- from the growth medium was observed for the *Synechococcus* PCC 7942 *psbO*$^-$ strain within the first 5 days of cell growth [63]. The *psbO*$^-$ strain has been reported to be more susceptible to photoinhibition [134,180]. In addition, in these 33 kDa polypeptide-less strains, the oxygen-evolving activity is inhibited by dark-adaptation of the cells with a half-life time of 10 min in *Synechococcus* PCC 7942 or 20 minutes in *Synechocystis* PCC 6803 *psbO*$^-$ strains. Oxygen evolution can be recovered by flash- or continuous illumination [39,63].

The decrease in oxygen evolution seems to be in part due to the inhibition of the S_3–S_0 transition since the rate of O_2-release from PSII is reduced [36,63]. In addition, oxygen evolution after light flashes showed a damped pattern which might indicate a higher number of misses. These results are difficult to interpret since the S-state distribution at the start of the measurement is not exactly known. Thermoluminescence measurements indicate a higher stability of the S_2 and S_3 states [36,223]. However, their interpretation is hampered by the fact that the glow curves exhibit an area of only about 25% that of the glow curves of the wild-type, indicating that they represent only a minor fraction of the PSII centres or that the manganese cluster in a large fraction of centres became reduced during the measurements by components outside of PSII.

The isolation of thylakoid membranes from the *Synechocystis* PCC 6803 *psbO*$^-$ strain has been reported [37,38]. For this 33 kDa polypeptide-less strain the oxygen-evolving activity could be maintained only in the presence of high concentrations of Cl^- which is in agreement with the behaviour of PSII from higher plants lacking the 33 kDa polypeptide.

In order to study function and binding of the 33 kDa polypeptide, site-directed mutagenesis was carried out on carboxylic residues of the spinach [195,196,198,199] and the *Synechocystis* PCC 6803 33 kDa protein [37,38]. Carboxylic amino acid residues are the most likely candidates to be involved in binding of manganese and calcium. Moreover, there are acidic amino acid residues involved in binding of the polypeptide to PSII (A.S., submitted, see also Refs. [97,199]).

The spinach protein and its variants carrying mutations in all conserved acidic amino acid residues (see Fig. 3) were expressed in *E. coli* [194] and reconstituted to $CaCl_2$-washed PSII. Some of the protein variants could not restore the activity up to the level obtained with the wild-type protein but this effect could be assigned to the protein variants having a lower binding affinity to PSII rather than to a slowing down of the S-cycle [198].

The strongest decrease in binding was observed when mutations were introduced at D109 and D157

[196,198]. Both aspartic acid residues were replaced by asparagine (N) and lysine (K). The variant D109N accumulated in *E. coli* to almost the same level as the wild-type 33 kDa polypeptide (17% of the periplasmic proteins, compared to 25% for the wild-type protein). However, the variant D109K accumulated only to 3% of the periplasmic proteins, indicating that either the folding of the protein variant is slowed down or that the mutation led to a different, less protease-resistant conformation of the 33 kDa polypeptide. Moreover, during purification the addition of 10% glycerol to the buffer was necessary. Whereas the variant D109N of the 33 kDa polypeptide had almost the same binding affinity as the wild-type protein, the variant showed an almost 10-fold lower binding affinity. The lower accumulation

A

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                    9                   28
Spinach         EGG.KRLTYD EIQSKTYLEV KGTGTANQCP TV.EGGVDSF AF.KPGKYTA
Pea             EGAPKRLTFD EIQSKTYLEV KGTGTANQCP TI.DGGVDSF SF.KPGKYNA
Arabidopsis     EGAPKRLTYD EIQSKTYMEV KGTGTANQCP TI.DGGSETF SF.KPGKYAG
Wheat           EGAPKRLTFD EIQSKTYMEV KGTGTANQCP TI.DGGVDSF PF.KAGKYEM
Tomato          EGVPKRLTYD EIQSKTYMEV KGTGTANQCP TI.EGGVGSF AF.KPGKYTA
Potato          EGVPKRLTFD EIQSKTYMEV KGTGTANQCP TI.NGGVDSF AF.KPGKYNA
Chlamy          ......LTFD EIQGLTYLQV KGSGIANTCP VL.ESGTTNL KELKAGSYKL
Euglena         ....ASLTYD ELQSLSYLEV KSSGIAGTCP VLADGVSSKL S.LKAGKYEI
Synechococcus   .ADLGTLTYD QI........ KDTGLANKCL SLKESARGTI PLEAGKKYAL
Synechocystis   .VDKSQLTYD DI........ VNTGLANVCP EISSFTRGTI EVEPNTKYFV
Anabaena        ..TRDILTYE QI........ RGTGLANKCP QLTETSRGSI PLDSSKSYVL
S.elongatus     ..AKQTLTYD DI........ VGTGLANKCP TLDDTARGAY PYDSSQTYRI
consensus       ------**** -*-------- --**-**-** ---------- -------*--

                  53        62                        86          97
Spinach         KKFCLEPTKF AVKAEGISKN SGPDFQNTKL MTRLTYTLDE IEGPFEVSSD
Pea             KKLCLEPTSF TVKSEGVTKN TPLAFQNTKL MTRLTYTLDE IEGPFEVSAD
Arabidopsis     KKFCFEPTSF TVKADSVSKN APPEFQNTKL MTRLTYTLDE IEGPFEVASD
Wheat           KKFCLEPTSF TVKAEGIQKN EPPRFQKTKL MTRLTYTLDE MEGPLEVRRR
Tomato          KKFCLEPTSF TVKAEGVSKN SAPDFQKTKL MTRLTYTLDE IEGPFEVSPD
Potato          KKFCLEPTSF TVKAEGVSKN SAPDFQKTKL MTRLTYTLDE IEGPFEVSPD
Chlamy          ENFCIEPTSF TVKEESQFKG GETEFVKTKL MTRLTYTLDA MSGSFKVGSD
Euglena         NNWCLEPSSF QVKLPPTEKQ QVTEFERTKL MTRLTYTLDA ISADLNVGGD
Synechococcus   TDLCLEPQEF FVKEEPGNKR QKAEFVPGKV LTRYTSSLDQ VYGDLALKAD
Synechocystis   SDFCMEPQEY FVKEEPVNKR QKAEYVKGKV LTRQTTSLEQ IRGSIAVGAD
Anabaena        KELCLEPTNF FVKEEPANKR QTAEFVAGKL LTRYTSTIDQ VSGDLKFNDD
S.elongatus     ARLCLQPTTF LVKEEPKNKR QEAEFVPTKL VTRETTSLDQ IQGELKVNSD
consensus       ---*-**--* -**------*- ----*---** -**-*-***- --*-------

                  104   109          121
Spinach         GTVKFEEKDG IDYAAVTVQL PGGERVPFLF TIKQLVAS.G KP........
Pea             GSVKFEEKDG IDYAAVTVQL PGGERVPFLF TIKQLVAS.G KP........
Arabidopsis     GSVNFKEEDG IDYAAVTVQL PGGERVPFLF TVKQLDAS.G KP........
Wheat           RTLKFEEKDG IDYAAVTVQL PGGERVAFLF TVKQLVAT.G KP........
Tomato          GTVKFEEKDG IDYAAVTVQL PGGERVPFLF TIKQLVAS.G KP........
Potato          GTVKFEEKDG IDYAAVTVQL PGGERVPFLF TIKQLVAS.G KP........
Chlamy          GSAELKEDDG IDYAATTVQL PGGERVAFLF TIKQFDGK.G TL........
Euglena         GSWTIQEKDG LDYAATTVQL AGGERVPFLF TIKNLLAK.G DA........
Synechococcus   GTVSFTEKGG IDFQAITVLL PGGEEVPFLF TVKGLVASTS EPA.TSINTS
Synechocystis   GTLTFKEKDG IDFQPITVLL PGGEEVPFFF TVKNFTGTT. EPGFTSINSS
Anabaena        SSLTFVEKDG LDFQAITVQL PGGERVPFLF TIKNLVAQT. QPGLSSLNTS
S.elongatus     GSLTFVEEDG IDFQPVTVQM AGGERIPLLF TVKNLVAST. QPNVTSITTS
consensus       **----*--* ***--***-* ********** ***------- -*--------
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Fig. 3. Sequence alignment of 33 kDa polypeptides from different organisms. The numbers indicate amino acid residues which have been mutagenised. Spinach [219]; pea [229] (the sequence of another member of the pea *psbO* gene family has been published too [230]; the following differences were found: 119R, 199V, 244R, 246N, 247H, 248R (numbering according to the spinach sequence)); *Arabidopsis* [110]; wheat [139], tomato [75], potato [221]; *Chlamydomonas reinhardtii* (Chlamy) [138]; *Euglena gracilis* Z: [206]; *Synechococcus* PCC 7942 (*Anacystis nidulans* R2) [120]; *Synechocystis* PCC 6803 [179]; *Anabaena* PCC 7120 [22]; *Synechococcus elongatus* [148].

B

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                                157         168
Spinach          .ESFSGDFLV PSYRGSSFLD PKGRGGSTGY DNAVALPAGG RGDEEELQKE
Pea              .DSFSGEFLV PSYRGSSFLD PKGRGASTGY DNAVALPAGG RGDEEELGKE
Arabidopsis      .DSFTGKFLV PSYRGSSFLD PKGRGGSTGY DNAVALPAGG RGDEEELVKE
Wheat            .DSFRP.FLV PSYRGSSFLD PKGRGGSTGY DNAGALPRGG RGDEEELAKE
Tomato           .ESFSGEFLV PSYRGSSFLD PKGRGGSTGY DNAVALPAGG RGDEEELQKE
Potato           .ESFSVDFLV PSYRGSSFLD PKGRGGSTGY DNAVALPAGG RGDEEELQKE
Chlamy           .DGIKGDFLV PSYRGSSFLD PKGRGGSTGY DNAVALPA.. RADAEELLKE
Euglena          .GQFLGQFDV PSYRGATFLD PKGRGGASGY DTAVALPASG ..DDEEYAKE
Synechococcus    TD.LRGGYRV PSYRTSNFLD PKARGLTTGY ESAVAIPSAG ..DAEDLTKE
Synechocystis    TD.FVGDFNV PSYRGAGFLD PKARGLYTGY DNAVALPSAA ..DKFRTNK.
Anabaena         TD.FEGTFKV PSYRGSAFLD PKGRGVVSGY DNAVALPAQA ..DDEDLTRT
S.elongatus      TD.FKGEFNV PSYRTANFLD PKGRGLASGY DSAIALP.QA K..EEELARA
consensus        ---*-*-*-* ****-*-*** **-**--*** *******--------*-*-*-

                                     212         224 226     234
Spinach          NNKNVASSKG TITLSVTSSK PETGEVIGVF QSLQPSDTDL GAKVPKDVKI
Pea              NNKSAASSKG KITLSVTQTK PETGEVIGVF ESIQPSDTDL .AKAPKDVKI
Arabidopsis      NVKNTAASVG EITLKVTKSK PETGEVIGVF ESLQPSDTDL GAKVPKDVKI
Wheat            NVKNASSSTG NITLSVTKSK PETGEVIGVF ESVQPSDTDL EA..PKDVKI
Tomato           NVKNTASLTG KITLSVTQSK PETGEVIGVF ESIQPSDTDL GAKVPKDVKI
Potato           NVKNTASLTG KITFTVTKSN PQTGEVIGVF ESIQPSDTDL GAKTPKDVKI
Chlamy           NVKITKALKG SAVFSVAKVD PVTGEIAGVF ESIQPSDTDL GAKPPKDIKV
Euglena          NSKSTAASVG TIAFKVAKVN AETGEIAGVF ESIQPSDTDL GAKVPKDIKT
Synechococcus    NVKRFVTGQG EISLAVSKVD GATGEVAGVF TAIQPSDTDM GGKEAVDVKL
Synechocystis    ..KETPLGKG TLSLQVTQVD GSTGEIAGIF ESEQPSDTDL GAKEPLDVKV
Anabaena         NVKRAEILNG KISLQIAKVD SSSGEIAGTF ESEQPSDTDL GADEPKEVKI
S.elongatus      NVKRFSLTKG QISLNVAKVD GRTGEIAGTF ESEQLSDDDM GAHEPHEVKI
consensus        --*------* ---*-*---- --*******-* -*-******* -*--*-****

Spinach          EGVWYAQLEQ ...
Pea              QGVWYAQLES ...
Arabidopsis      QGVWYGQLE. ...
Wheat            QGVWYAQLES N..
Tomato           QGIWYAQLE. ...
Potato           QGIWYAQLES ...
Chlamy           TGLWYAQLK. ...
Euglena          SGVWYAQISP SK.
Synechococcus    VGQFYGRIEP ADA
Synechocystis    RGIFYGRVDT DV.
Anabaena         RGIFYARVE. ...
S.elongatus      QGVFYASIEP A..
consensus        -*-**--*-- ---
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Fig. 3 (continued).

of the variant D109K in *E. coli*, the stabilising effect of glycerol and the lower binding affinity to PSII indicate that this variant has a different conformation than the wild-type 33 kDa polypeptide.

The variant D157N and D157K both exhibit a lower binding affinity than the wild-type protein. Both variants accumulated in *E. coli* to a level similar to the wild-type protein, indicating that the mutations did not lead to a significant increase in protease sensitivity of the 33 kDa polypeptide. Therefore, D157 is a good candidate to be involved in binding of the 33 kDa polypeptide to PSII. Replacement of D159 in the 33 kDa polypeptide of *Synechocystis* PCC 6803, which is the equivalent residue to D157 in the spinach protein, by asparagine led to a phenotype which is most easily explained by a lowered binding of the protein variant [38] and is therefore consistent with the results obtained with the spinach polypeptide.

Particularly remarkable are the variants of the 33 kDa polypeptide D212N and D212K, where the aspartic acid residue 212 was replaced by glutamine and lysine, respectively. These protein variants accumulated only to a low level in *E. coli*, $< 1\%$ of the

periplasmic protein fraction, compared to 25% for the wild-type protein. During purification via ion exchange chromatography even in the presence of 20% glycerol the protein variants were eluted from the column over a wide range of the NaCl gradient, indicating a very heterogeneous population of protein conformations. However, the protein variant D212N could be purified by adopting an alternative strategy. A histidine tail was introduced at the N-terminus of the 33 kDa polypeptide [193] which allowed the use of immobilised metal ion affinity chromatography for purification of the recombinant protein. The protein variant D212N purified by this strategy was able to restore the oxygen-evolving activity to a level comparable to that of the wild-type protein (A.S., unpublished). This indicates that a subtle change like the replacement of an aspartic acid residue by asparagine is able to destabilise the conformation of the 33 kDa polypeptide. This destabilisation seems to have no effect on the binding affinity of the 33 kDa polypeptide. A variant of the 33 kDa polypeptide with probably similar properties is A235V [19]. This variant shows a cold-sensitivity during reconstitution to PSII. Both, D212 and A235 are located at the beginning of predicted β-sheet structures [236] which might become destabilised by the introduced mutations.

Mutations of the spinach or *Synechocystis* PCC 6803 33 kDa polypeptide at residue D9 led to no reduction of the oxygen-evolving activity or change in the binding constant of the protein to PSII [195,196,199]. (The *Synechocystis* PCC 6803 mutant D9N was previously reported to lack binding of the mutagenised 33 kDa polypeptide but this was due to a second-site mutation [R. Burnap, personal communication].) In recent PSII cross-linking experiments using EDC, three cross-linked products due to intramolecular cross-linking of the 33 kDa polypeptide could be detected (A.S., submitted, see also [199]). The same cross-linking method using $CaCl_2$-washed PSII reconstituted with the protein variant D9N revealed that in this variant only two intramolecular cross-linked products could be detected. Therefore, the residue D9 was suggested to be involved in one of the intramolecular salt bridges [199].

Site-directed mutagenesis of the disulphide bridge of the *Synechocystis* PCC 6803 protein confirmed the results of in vitro studies [217] in showing that the disulphide bridge is essential for binding of the 33 kDa polypeptide to PSII [38].

4.2.3. Metal binding properties

Several regions of the 33 kDa polypeptide show homologies to metal binding sites. Residues 18 to 33 show some homology to part of the metal binding site in a bacterial manganese superoxide dismutase [168]. However, the metal binding residue in the superoxide dismutase, an aspartic acid side chain, is not conserved in the 33 kDa polypeptide. Instead, the 33 kDa polypeptide contains a cysteine residue at this position and this is unlikely to be involved in manganese ligation since this cysteine residue and C51 of the polypeptide form a disulphide bridge [114,217].

It has been suggested that the disulphide bridge in the 33 kDa polypeptide may be important in oxygen evolution since PSII could be reversibly inhibited by treatment with dithioerythritol [95]. An alternative explanation is that the two cysteine residues of the D2 protein localised in the putative loop between helix 1 and 2 [143] form a disulphide bridge and that the reduction of this disulphide bridge led to the inhibition of oxygen evolution. Further studies are required in order to clarify this point.

Several Ca^{2+} binding sites have been suggested to be located on the 33 kDa polypeptide of higher plants because of sequence similarities to Ca^{2+}-binding proteins with known structures [49,229]. However, none of these suggested sites is totally conserved in cyanobacteria. The number of Ca^{2+} sites in higher plant PSII-enriched membranes has been determined to be 2 (in earlier studies sometimes 3, see Refs. [2,40,54]). Only one Ca^{2+} has been found in PSII core preparations [58,82] and cyanobacterial PSII [104]. This is consistent with findings that only one Ca^{2+} is involved in oxygen evolution [40,174]. The second Ca^{2+} binding site seems to be located on an antenna protein [82,231], possibly CP29 [96]. An effect of Ca^{2+} on oxygen evolution was also observed in the absence of the 33 kDa polypeptide, indicating the presence of a Ca^{2+}-binding site involved in oxygen evolution on the intrinsic polypeptides [153,171]. However, this site might be different in the presence and absence of the 33 kDa protein. It was suggested that the Ca^{2+}-binding site involved in oxygen evolution might be located in

between the extrinsic 33 kDa protein and an intrinsic PSII subunit [244]. The idea of an involvement of the 33 kDa protein in Ca^{2+}-binding was supported by the finding that the *psbO*$^-$-strain of *Synechocystis* PCC 6803 is much more sensitive to the depletion of Ca^{2+} from the growth medium than the wild-type [63,180]. Under the assumption that a depletion of Ca^{2+} in the growth medium corresponds to a lowered intramolecular Ca^{2+} level, this observation might be an indication of a lower binding affinity of PSII for Ca^{2+} in the absence of the 33 kDa protein. However, it is also possible that in the absence of the 33 kDa protein an elevated level of Ca^{2+} protects the manganese cluster from being over-reduced or reduced manganese from being released from PSII.

Recent studies on the effect of the 33 kDa protein on Ca^{2+}-binding revealed that it is bound to PSII with high affinity [43]. Moreover, as already observed in PSII lacking the 23 and 16 kDa proteins but retaining the 33 kDa protein, Ca^{2+} cannot be extracted by chelators in the S_1 and only partially in the S_2-state of the manganese cluster [197]. The most rapid release of Ca^{2+} seems to occur in the S_3-state, whether or not the 33 kDa polypeptide is present. Therefore, the 33 kDa polypeptide seems not to be involved in Ca^{2+}-binding.

Ädelroth et al. [2] found by equilibrium dialysis that only 0.3 Ca^{2+} per PSII could be bound after removal of the 33 kDa polypeptide. This might be interpreted as a drastic reduction of the binding affinity for Ca^{2+} due to the removal of the 33 kDa polypeptide. However, in their preparation no Ca^{2+}-depletion was carried out prior to the equilibrium dialysis. From the recent finding of the tight binding of Ca^{2+} even in the absence of the 33 kDa protein [42,197], it seems possible that on the time scale of the measurements the majority of the PSII reaction centres which still contained Ca^{2+} did not exchange the metal ion. The 0.3 Ca^{2+} per reaction centre might correspond to the fraction of centres which lost the Ca^{2+} during sample preparation. This would be consistent with the fit of the data to 0.2 Ca^{2+} with a binding constant of 100 μM, which is in the same order of magnitude as in PSII carrying the 33 kDa polypeptide.

In conclusion, there is little possibility for the 33 kDa polypeptide to contain the Ca^{2+}-binding site which is involved in oxygen evolution. If the 33 kDa polypeptide contains a Ca^{2+}-binding site, it is of rather low affinity and has thus far escaped detection. Since in the absence of the 33 kDa polypeptide the Ca^{2+}-binding site in the S_1-state of the manganese cluster is not accessible to EGTA [197], it is also unlikely that the Ca^{2+}-binding site is shared between an intrinsic PSII subunit and the 33 kDa protein.

The 33 kDa polypeptide does not seem to be involved in manganese binding either, although the available data do not exclude the possibility that a manganese ligand from the 33 kDa polypeptide is replaced by Cl^- when the 33 kDa polypeptide is absent. The 33 kDa polypeptide is involved in stabilisation of the manganese cluster and protects it from the attack by endogenous reductants. It also effects the conformation of the lumenal side of intrinsic PSII subunits which are responsible for binding of the 23 and 16 kDa polypeptide (see Section 4.1.1).

4.2.4. The function of the 33 kDa polypeptide in photoactivation

Photoactivation is the light-induced development of oxygen-evolving activity in PSII. In this process manganese ions become ligated to preformed PSII due to photooxidation of Mn^{2+}. Photoactivation in PSII can occur in vivo (at least in cyanobacteria, see Section 4.2.2) and in vitro [214,215], without the 33 kDa polypeptide. However, in the in vitro experiments maximal activity could be obtained with 300 mM Cl^- in the presence of the 33 kDa polypeptide, but 1 M Cl^- was required in its absence [157]. Besides a stabilisation of the formed manganese cluster by a Cl^--concentration higher than 100 mM [159], there seems to be an additional effect of the Cl^--ion in photoactivation which can compensate for the absence of the 33 kDa polypeptide. This can be either due to the fact that Cl^- acts as a chaotroph at high concentrations disrupting hydrophobic interactions [157], or due to a charge effect where Cl^- replaces the 33 kDa polypeptide which is rich in negatively charged amino acid residues.

The *psbO* gene has been deleted in mutants of *Synechocystis* PCC 6803 carrying mutations in the *psbA* gene encoding the D1 protein [45]. Time-resolved fluorescence measurements after flash-induced charge separation were carried out in order to determine the number of centres which contained photooxidisable manganese. In *psbO*$^+$-strains, it was found

that mutagenesis of aspartic acid 170, glutamic acid 333, and histidines 332 and 337 led to a loss of oxygen evolution and a drastic decrease of manganese photooxidation [44]. When, however, the same mutants were generated in a *psbO*$^-$-background, higher levels of manganese photooxidation were found [45]. It was concluded that the 33 kDa polypeptide hinders the access of Mn^{2+} to the high affinity binding site where it can become photooxidised. For spinach PSII lacking the manganese cluster, it has been shown that this polypeptide has no influence on the oxidation of Mn^{2+} by Tyr_z^+ [87]. Therefore, the 33 kDa polypeptide seems to be involved in (a) later step(s) of photoactivation.

4.3. The function of the low potential cytochrome c-550 and the 9–12 kDa polypeptide

The association of cytochrome *c*-550 and a 9 kDa polypeptide with PSII was first found in *Phormidium laminosum* [29,209], although at the time it was not clear whether the cytochrome was a true subunit of PSII. Treatment of PSII of this strain with 1 M NaCl or incubation in a glycerol-free medium led to a decrease in oxygen evolution and to a release of a 9 kDa polypeptide [209]. The lost activity could be partially restored by rebinding of the protein [184].

Recently, cytochrome *c*-550 has been found in *Synechococcus vulcanus* [202], *Synechococcus* PCC 7002 [165], *Synechocystis* PCC 6803 [102,205], and the red algae *Cyanidium caldarium* [61]. A refined preparation of PSII from *S. vulcanus* and *C. caldarium* contained the cytochrome *c*-550 and a 12 kDa polypeptide homologue of the 9 kDa polypeptide of *P. laminosum*. Unlike the 9 kDa polypeptide of *P. laminosum*, the 12 kDa polypeptide of *S. vulcanus* and *C. caldarium* could not be released by 1 M NaCl or incubation in glycerol-free buffer but only by treatment with 1 M $CaCl_2$ [202], urea/NaCl [61] or 1 M Tris (pH 8.5) [61,202], with concomitant release of the 33 kDa polypeptide and cytochrome *c*-550. The release of cytochrome *c*-550 and the 12 kDa polypeptide was accompanied by a loss of activity. $CaCl_2$-washed PSII of *S. vulcanus* showed only 10% of the activity of intact PSII (in the presence of 10 mM Ca^{2+} and 120 mM Cl^-) [203]. Rebinding of the 33 kDa polypeptide restored 47% of the activity, additional rebinding of cytochrome *c*-550 increased the activity to 57%, and after rebinding of all three polypeptides 79% of the activity was reached. All activity measurements of reconstituted PSII were performed in the presence of 10 mM Ca^{2+} and 30 mM Cl^-. In the absence of the 33 kDa polypeptide, addition of either the cytochrome or the 12 kDa polypeptide or both to $CaCl_2$-washed PSII did not restore any activity. Whereas the cytochrome could rebind to PSII in the absence of the 33 kDa polypeptide, although with lower affinity, the 12 kDa polypeptide could not. The 12 kDa polypeptide required both of the other extrinsic proteins for rebinding [203]. Measurements of oxygen evolution with non-saturating light intensities showed that the lack of the cytochrome and the 12 kDa polypeptide did not result in a reduction of the S-cycle turnover rate but rather to a lowering in quantum yield of PSII. The activity was stimulated in all preparations by divalent cations whatever the composition of the extrinsic polypeptides. Ca^{2+} is more effective than Mg^{2+} but a significant stimulation of activity also occurred with Mg^{2+}, indicating a different, more structural role of the divalent cation in PSII from *S. vulcanus* and other thermophilic cyanobacteria [178,184,209] or *C. caldarium* [61] compared to Ca^{2+} in PSII of higher plants.

Recently cytochrome *c*-550 has been found in the mesophyllic cyanobacteria *Synechocystis* PCC 6803 [102,132,205] and *Synechococcus* PCC 7002 [165] and the genes were cloned. The two published sequences for the *psbV* genes from *Synechocystis* PCC 6803 differ in their 5′-end. Only Shen et al. [205] found a coding region for a thylakoid transfer peptide. However, the gene of cytochrome *c*-550 from *Synechococcus* PCC 7002 was also found to contain the coding region for a signal peptide. Moreover, the sequence found by Kang et al. [102] did not contain a typical start codon. The DNA sequence in Ref. [102] differs from the sequence in Ref. [205] only in one additional base causing a frame shift in the coding region for the thylakoid transfer peptide.

A cytochrome *c*-550 was found in a PSII preparation from *Synechocystis* PCC 6803 [132]. However, the most pure but still oxygen-evolving PSII preparation from this cyanobacterial strain does not contain this subunit [218], suggesting a rather loose association with the photosystem (see also Section 2).

Cytochrome *c*-550 could be removed from thyl-

akoid membranes of *Synechococcus* PCC 7002 by treatment with 0.1% Triton X-100 [165]. Rebinding of cytochrome *c*-550 stabilises PSII against heat inactivation [165]; however, the effect of the release of the protein on oxygen evolution was minor and could not be restored by rebinding of the protein.

In *Synechocystis* PCC 6803 the role of cytochrome *c*-550 was studied by deletion of the gene [205]. The deletion strain showed a doubling time which was more than twice that of the wild-type when photoautotrophically grown and the number of PSII reaction centres was only half of the wild-type. On a chlorophyll basis the oxygen-evolving activity of the mutant was found to be around 42% of the wild-type. Therefore, the major reduction of the oxygen-evolving activity is due to a decreased stability of the PSII reaction centre and the activity per reaction centre is not or only slightly diminished.

A double-deletion strain lacking the *psbV* and *psbO* genes did not grow photoautotrophically and contained only 20% of PSII compared to the wild-type strain [200]. The initial rate of oxygen evolution was only 8% of the wild-type and declined rapidly.

In summary, cytochrome *c*-550 seems to be necessary only for the conformational integrity of PSII rather than being directly involved in the enzyme activity itself. In mesophyllic cyanobacteria, the influence of the protein on oxygen evolution seems to be negligible. Only the stability of PSII is affected. In PSII from *S.vulcanus*, and perhaps also in PSII from other thermophilic cyanobacteria, the influence seems to be stronger since the quantum yield of PSII is reduced. This could result from a reduction of (an) electron transfer step(s) allowing some charge recombination to occur. Alternatively, the release of cytochrome *c*-550 could induce partial disconnection of antenna proteins from the reaction centre.

5. The binding sites of the extrinsic polypeptides

5.1. The binding site of the 33 kDa polypeptide

The region of the 33 kDa protein that is involved in binding to PSII has been suggested to lie near its N-terminus since removal of the first 16 or 18 amino acid residues by limited proteolysis led to a loss in its binding capability [56]. However, a recent study suggests that the deletion of the N-terminal part of the protein results in a conformational change of the binding region of the 33 kDa polypeptide to PSII [199].

After treatment of PSII with trypsin, a 15 kDa fragment of the 33 kDa polypeptide was still bound to PSII. This fragment was supposed to contain the binding site of the polypeptide to the PSII [226]. However, it cannot be excluded that more fragments remained bound to PSII and were too small to be detected by gel electrophoresis.

The binding sites of the 33 kDa polypeptide at PSII have been studied by accessibility studies, immunoprecipitation and cross-linking. In addition its location has been assigned in recent electron microscope pictures of PSII. As a result multiple binding sites have been suggested.

5.1.1. Binding of the 33 kDa polypeptide to D1 / D2 / cyt b-559 / PsbI

Removal of the 33 kDa protein from inside-out thylakoid membrane vesicles led to an increased accessibility of the D1 reaction centre subunit to antibodies raised against the predicted lumenal loops of this protein [191]. Moreover, it has been reported that after cross-linking of the 33 kDa protein a manganese-containing protein complex consisting of the 33 kDa protein and the D1/D2 reaction centre heterodimer could be isolated [140]. The isolated D1/D2/cyt *b*-559/*Psb*I complex has also been reported to be able to bind the 33 kDa polypeptide [34,76], but the specificity of the binding could not be proven.

5.1.2. The binding of the 33 kDa polypeptide to cytochrome b-559

Cross-linking studies with PSII complexes lacking LHCII with hexamethylene diisocyanate, a cross-linking agent with a spacer length of 11 Å, led to covalent binding of the 33 kDa polypeptide with the α-subunit of cytochrome *b*-559 and the *Psb*I protein [62]. Trypsin treatment of PSII in the presence of the 33 kDa polypeptide did not affect the cytochrome *b*-559. However, in its absence the α-subunit of the cytochrome is degraded [211]. Recently it was found that the C-terminus of the α-subunit of cytochrome *b*-559 could be deleted without affecting normal function of PSII [212]. This result is not consistent

with the idea that the 33 kDa polypeptide is bound to cytochrome *b*-559 (n.b., the β-subunit most likely has only a single amino acid residue exposed on the lumenal side and thus makes an unlikely cross-linking partner). However, the results of Tae and Cramer [212] have been questioned [207].

5.1.3. The binding of 33 kDa polypeptide to CP47

There are several lines of evidence indicating that the 33 kDa polypeptide binds to the chlorophyll *a*-binding protein CP47: (1) The extrinsic polypeptide shields CP47 from proteolytic attack [31], although the increase in accessibility to external proteases was reported to be more pronounced for CP43 [97] (see below). (2) Removal of the 33 kDa polypeptide and two out of four manganese ions was necessary before lysine residues on the large lumenal loop between the putative transmembrane helices 5 and 6 of CP47 could be labelled with NHS-biotin [32,66]. (3) Deletion of short parts (4–13 amino acid residues) of the large hydrophilic loop of CP47 led to a decreased binding of the 33 kDa polypeptide, particularly in the region of residues 373-392 [57,73,74,78]. (4) The 33 kDa polypeptide and CP47 could be cross-linked by EDC, a water-soluble carbodiimide [32,59]. In contrast to all other cross-linkers, EDC connects only proteins which are in Van der Waal's contact to each other. Since EDC is highly substrate-specific (carboxylic acid side chains become covalently bound to primary amines), only a few interacting sites are cross-linked by EDC. The cross-linked sites were mapped by chemical cleavage of one of the two cross-linked products and subsequent N-terminal sequencing of the isolated peptides. It was found that a residue on the N-terminal fragment of 88 amino acid residues of the 33 kDa polypeptide was cross-linked to a residue on the large lumenal loop between the putative transmembrane helices 5 and 6 of CP47, probably in the region between amino acid residues 360 and 420 [167]. Recently, it was found that only the carboxylic acid side chains of the 33 kDa polypeptide were involved in the cross-linking to CP47 (A.S., submitted). From this result together with the results of the mutational analysis of CP47 and the mapping of the cross-linking sites by N-terminal sequencing [167], it can be concluded that (one of) the lysine residue(s) of CP47 involved in cross-linking to the 33 kDa polypeptide may be K389.

5.1.4. Binding of the 33 kDa polypeptide to CP43

Binding of the 33 kDa polypeptide to CP43 has also been suggested. Removal of the 33 kDa polypeptide increases the susceptibility of CP43 to proteolytic attack, whereas the susceptibility of CP47 was only slightly increased [98].

5.1.5. The binding of the 33 kDa polypeptide in mutants of Synechocystis PCC 6803 lacking intrinsic PSII subunits

Deletion of all three copies of the *psbA* gene encoding the D1 reaction centre subunit led to a strong decrease in the subunits D2 and the chlorophyll *a*-binding protein CP47 in the thylakoid membrane. However, the 33 kDa polypeptide remained tightly bound to the membrane [164]. This extrinsic polypeptide could not be released by treatment with 1 M Tris and concomitant sonication. Therefore, it was suggested that one or more of the remaining transmembrane subunits, such as cytochrome *b*-559 or CP43, might be involved in binding of the 33 kDa polypeptide. Ikeuchi et al. [94] found that the 33 kDa polypeptide was still present in thylakoid membrane preparations from mutants of *Synechocystis* PCC 6803 lacking the gene(s) for either D1, CP47, CP43, D2 + CP43 or the *psbEFLJ* operon or expressing an extremely unstable mutant of D2, even after treatment with 0.1% dodecyl maltoside. These findings are in conflict with recent findings showing that the 33 kDa polypeptide can be released from thylakoid membranes of cells containing short deletions in the *psbB* gene encoding CP47 by treatment with 0.03% dodecyl maltoside [73]. The apparent discrepancy between Refs. [73] and [94] might be explained by the fact that in Ref. [73] thylakoid membranes have been used for the Western blots which were stored frozen, whereas in Ref. [94] the membranes used were freshly prepared (J.R. Shen, personal communication). Freezing and thawing of the thylakoid membranes might have disrupted the membrane vesicles so that after the following washing with detergent, weakly bound 33 kDa protein was released from the membrane. On the other hand, the treatment of freshly prepared thylakoid membranes with 0.1% dodecyl maltoside might not have allowed the solubilisation

of the thylakoid membrane at the chlorophyll-to-detergent ratio applied so that unbound 33 kDa polypeptide was not released from the thylakoid membrane vesicles (J.R. Shen, personal communication). However, although the discrepancy to Ref. [164] remains, it seems likely that the 33 kDa protein requires an intact PSII reaction centre for tight binding. The same was found for PSII from higher plants (reviewed in Ref. [11]).

5.1.6. The location of the 33 kDa protein by electron microscopy

Recently low resolution projection maps of PSII were obtained (see Section 2) where certain regions were assigned to PSII subunits. According to this assignment, the 33 kDa polypeptide is located close to the D1/D2 reaction centre subunits and CP47 [21].

In conclusion, the main binding site for the 33 kDa polypeptide seems to be located on CP47. Other subunits cannot be excluded from also being involved in binding but further investigations are necessary to prove this.

5.2. The binding sites of the 23 kDa and the 16 kDa polypeptides

Like the N-terminus of the 33 kDa polypeptide, the N-terminus of the 23 and the 16 kDa polypeptide can be removed by limited proteolysis [118,149]. Whereas the removal of 8 residues from the N-terminus of the 23 kDa polypeptide led only to a moderate decrease of its binding affinity to PSII, removal of 13 residues from the N-terminus of the 16 kDa polypeptide led to a total loss of its binding to PSII. Therefore, it has been suggested that the N-terminus of the 16 kDa polypeptide might be involved in binding to PSII.

There is some discrepancy in the literature on whether the 33 kDa polypeptide is absolutely required for the binding of the 23 and 16 kDa polypeptides. It was found that the 23 kDa polypeptide could not be rebound to Tris-washed inside-out thylakoid membrane vesicles [9]. The same has been observed with PSII-enriched membranes after removal of the 33 kDa polypeptide by $CaCl_2$-treatment [105]. Therefore, it was suggested that the 33 kDa polypeptide provides the binding site for the 23 kDa polypeptide but it could not be excluded that binding of the 33 kDa polypeptides creates the binding site(s) for the 23 kDa polypeptide due to a conformational change in intrinsic membrane protein(s). In a study where the extrinsic polypeptides were removed by urea/NaCl treatment, the 23 kDa polypeptide could be rebound to PSII, although only in substoichiometric amounts and the affinity was lowered [155]. The finding that the 33 kDa polypeptide could be extracted by water-butanol phase partitioning [238], or partially extracted by treatment with Hg^{2+} [16] without concomitant release of the other extrinsic polypeptides, also supports the idea of separate binding sites for the 33 and 23 kDa polypeptides. Moreover, in a mutant of *Chlamydomonas reinhardtii* lacking the ability to synthesise the 33 kDa polypeptide, the 23 and 16 kDa polypeptides are still bound to the thylakoid membrane [53].

In a mutant of the same strain, which is not able to synthesise the 23 kDa polypeptide, the 16 kDa polypeptide was not found to be bound to the thylakoid membrane [53], indicating that the 16 kDa polypeptide is bound to PSII via the 23 kDa polypeptide. Again, it cannot be excluded that the binding site of the 16 kDa polypeptide is located on an intrinsic subunit of PSII which changes its conformation due to binding of the 23 kDa polypeptide to PSII.

In mutants of *Chlamydomonas reinhardtii*, which are unable to synthesise the D1 reaction centre subunit or CP43, no functional PSII could be observed. However, immuno-gold labelling of the 23 kDa protein in the D1-less mutant revealed the protein mainly located in the stacked region of the thylakoids, whereas the protein was found randomly distributed in the thylakoids of the CP43-less strain [53]. Consequently, it has been suggested that CP43 might provide a binding site for the 23 kDa protein.

In the early experiments using EDC cross-linking, no cross-linked product involving the 23 kDa (or 16 kDa) polypeptide was found. This was somewhat surprising since the two extrinsic polypeptides can be released from PSII by washing with 1 M NaCl, indicating that their binding was mainly of an electrostatic nature. Recently it was discovered that the interacting sites are not accessible to EDC (A.S., unpublished). In experiments where carboxylic acid

groups of PSII lacking the 23 and 16 kDa polypeptides were activated with EDC and Sulfo-NHS before rebinding the 23 kDa polypeptide, cross-linking of the 23 kDa polypeptide to cytochrome *b*-559 and other intrinsic PSII subunits occurred (A.S., unpublished).

Cross-linking of the 23 kDa polypeptide to the 33 and the 16 kDa polypeptides using activated dicarboxylic acids with a length of 6 to 11 Å has been reported, indicating that the three extrinsic polypeptides must be located close to each other [60].

Early studies suggested the involvement of a 22 and a 10 kDa subunit in the binding of the 23 kDa polypeptide [130,131]. During the 1980's, increasingly smaller complexes of PSII still capable of oxygen evolution were prepared. The smallest complex still able to bind the 23 and 16 kDa polypeptides consisted of the D1 and D2 proteins, cytochrome *b*-559, CP47, CP43, CP29, the intrinsic 22 kDa protein and the extrinsic 33 kDa polypeptide [69,142]. However, the 10 kDa and 22 kDa proteins, as well as CP29, can be removed from PSII without affecting the binding of the 23 and 16 kDa polypeptides [146,147]. Therefore, the loss of binding ability for the 23 and 16 kDa polypeptides in an oxygen-evolving complex consisting of the D1 and D2 proteins, cytochrome *b*-559, CP47, CP43 and the 33 kDa extrinsic polypeptide might be due to a conformational change within the complex rather than due to the absence of the intrinsic subunit(s) involved in their binding in vivo.

The exact binding sites for the 23 and 16 kDa polypeptides still remains to be elucidated since all published data constitute rather indirect evidence for binding to a certain subunit. It is likely that they are bound to intrinsic subunits of PSII and not to the 33 kDa polypeptide.

5.3. The binding sites of the low potential cytochrome c-550 and the 9–12 kDa polypeptide

The binding sites of cytochrome *c*-550 and the 12 kDa polypeptide of *Synechococcus vulcanus* have been studied by cross-linking using EDC [83]. Cytochrome *c*-550 could be cross-linked to the reaction centre subunit D2 as well as to the 33 kDa and the 12 kDa polypeptide. The 12 kDa polypeptide was cross-linked to the cytochrome only. In addition, cross-linked products consisting of D2, cyt *c*-550 and the 12 kDa polypeptide as well as of the 33 kDa polypeptide, cyt *c*-550 and the 12 kDa polypeptide were found. Since no cross-linked products of the 12 kDa and the 33 kDa polypeptide and the 12 kDa polypeptide and D2 were found, it was assumed that the 12 kDa polypeptide binds only to cyt *c*-550.

6. Conclusions

Two main aspects of the 33, 23 and 16 kDa extrinsic polypeptides have been discussed in this review. One was their biogenesis and import into the thylakoid lumen, the other their function. Enormous progress was made in the last few years on the study of the import of the proteins. A new import system was discovered which is fundamentally different from the bacterial-type, Sec-related translocation system. The 23 and 16 kDa polypeptides, as well as PSII-T and PsaN and probably many other yet unknown lumenal proteins, are transported by this import system into the thylakoid lumen.

The biochemical studies on the extrinsic polypeptides, especially the dissociation and reconstitution experiments have provided much information concerning the function of Ca^{2+} and Cl^- in oxygen evolution, although the function of Cl^- is still rather enigmatic. The biochemical approach has better defined the function of the 23 and 16 kDa polypeptides: the 23 kDa polypeptide prevents Ca^{2+} from being released from PSII during the enzyme turn-over; both polypeptides increase the binding affinity of Cl^- for PSII; they protect the manganese complex from attack by reductants and other compounds; they stabilise the manganese cluster. The role of the 33 kDa polypeptide remains more unclear. All findings are consistent with the following simple interpretation: it stabilises the manganese complex by stabilising the protein(s) involved in manganese binding and is not involved in binding of an inorganic cofactor such as Ca^{2+} or Mn. However, one could imagine a quite more specific role in oxygen evolution including that the protein provides ligands to manganese but this remains to be proven. A clear answer can probably be given only by a high resolution crystal structure of oxygen-evolving PSII.

7. Note added after the revised manuscript was finished

The following publications appeared after the manuscript was finished. Nakazato et al. (1996, J. Mol. Biol. 257, 255–232) reported the isolation of an oxygen-evolving PSII core complex lacking the intrinsic 22 kDa polypeptide but retaining the 23 and 16 kDa extrinsic polypeptides, confirming that the 22 kDa polypeptide is not involved in the binding of the latter. Betts et al. (1996, Biochim. Biophys. Acta 1274, 135–142) reported that mutagenesis of the two cysteines of the 33 kDa polypeptide did not prevent binding of the protein to PSII and had no influence on oxygen evolution. The same authors reported also on a cold-sensitive assembly mutant of the 33 kDa protein (1996, Biochemistry 35, 6302–6307).

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References

[1] Abramovicz, D.A. and Dismukes, G.C. (1984) Biochim. Biophys. Acta 765, 318–328.
[2] Ädelroth, P., Lindberg, K. and Andréasson, L.-E. (1995) Biochemistry 34, 9021–9027.
[3] Åkerlund, H.-E. and Jansson, C. (1981) FEBS Lett. 124, 229–232.
[4] Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10.
[5] Akabori, K., Imaoka, A. and Toyoshima, Y. (1984) FEBS Lett. 173, 36–40.
[6] Alt, J. and Herrmann, R.G. (1984) Curr. Genet. 8, 551–557.
[7] Anderson, C.M. and Gray, J. (1991) FEBS Lett. 280, 383–386.
[8] Andersson, B., Critchley, C., Ryie, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. 168, 113–117.
[9] Andersson, B., Larsson, C., Jansson, C., Ljungberg, U. and Åkerlund, H.-E. (1984) Biochim. Biophys. Acta 766, 21–28.
[10] Andersson, B. and Styring, S. (1991) in Current topics in Bioenergetics (Lee, C.P., ed.), Vol. 16, pp. 1–81, Academic Press, New York.
[11] Aro, E.-M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta 1143, 113–134.
[12] Babcock, G.T., Barry, B.A., Debus, R.J., Hoganson, C.W., Atamian, M., McIntosh, L., Sithole, I. and Yocum, C.F. (1989) Biochemistry 28, 9557–9565.
[13] Barber, J. (1984) Trends Biochem. Sci. 9, 79–80.
[14] Bennoun, P., Diner, B.A., Wollmann, F.-A., Schmidt, G. and Chua, N.H. (1981) in Photosynthesis III (Akoyunoglou, G., ed), pp. 839–849, Balaban, Philadelphia.
[15] Berghöfer, J., Karnauchov, I., Herrmann, R.G. and Klösgen, R.B. (1995) J. Biol. Chem. 270, 18341–18346.
[16] Bernier, M. and Carpentier, R. (1995) FEBS Lett. 360, 251–254.
[17] Betts, S.D., Hachigian, T.M., Pichersky, E. and Yocum, C.F. (1994) Plant Mol. Biol. 26, 117–130.
[18] Betts, S.D., Ross, J.R., Pichersky, E. and Yocum, C.F. (1996) in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. II, pp. 385–388.
[19] Betts, S.D., Ross, J.R., Pichersky, E. and Yocum, C.F. (1996) in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. II, pp. 381–384.
[20] Bockholt, R., Masepohl, B. and Pistorius, E.K. (1991) FEBS Lett. 294, 59–63.
[21] Boekema, E.J., Hankamer, B., Bald, D., Kruip, J., Nield, J., Boonstra, A.F., Barber, J., Rögner, M. (1995) Proc. Natl. Acad. Sci. USA 92, 175–179.
[22] Borthakur, D. and Haselkorn, R. (1989) Plant Mol. Biol. 13, 427–439.
[23] Boussac, A. and Rutherford, A.W. (1988) FEBS Lett. 236, 432–436.
[24] Boussac, A. and Rutherford, A.W. (1988) Biochemistry 27, 3476–3483.
[25] Boussac, A. and Rutherford, A.W. (1990) FEBS Lett. 277, 69–74.
[26] Boussac, A., Zimmermann, J.-L. and Rutherford, A.W. (1989) Biochemistry 28, 8984–8989.
[27] Boussac, A., Zimmermann, J.-L., Rutherford, A.W. and Lavergne, J. (1990) Nature 347, 303–306.
[28] Bovy, A., De Vrieze, G., Borrias, M. and Weisbeek, P. (1992) Mol. Microbiol. 6, 1507–1513.
[29] Bowes, J.M., Stewart, A.C. and Bendall, D.S. (1983) Biochim. Biophys. Acta 725, 210–219.
[30] Bricker, T.M. (1992) Biochemistry 31, 4623–4628.
[31] Bricker, T.M. and Frankel, L.K. (1987) Arch. Biochem. Biophys. 256, 295–301.
[32] Bricker, T.M., Odom, W.R. and Queirolo, C.B. (1988) FEBS Lett. 231, 111–117.
[33] Briggs, L.M., Pecoraro, V.L. and McIntosh, L. (1990) Plant Mol. Biol. 15, 633–642.
[34] Brody, S.S. (1988) Z. Naturforsch. 43c, 727–730.
[35] Burnap, R.L. and Sherman, L.A. (1991) Biochemistry 30, 440–446.

[36] Burnap, R.L., Shen, J.-R., Jursinic, P.A., Inoue, Y. and Sherman, L.A. (1992) Biochemistry 31, 7404–7410.
[37] Burnap, R.L., Shen, J.-R., Jursinic, P.A., Qian, M., Inoue, Y. and Sherman, L.A. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. 2, pp. 179–182 Kluwer, Dordrecht.
[38] Burnap, R.L., Quian, M., Shen, J.-R., Inoue, Y. and Sherman, L.A. (1994) Biochemistry 33, 13712–13718.
[39] Burnap, R.L., Quian, M. and Pierce, C. (1996) Biochemistry 35, 874–882.
[40] Cammarata, K. and Cheniae, G.M. (1987) Plant. Physiol. 84, 587–595.
[41] Chaddock, A.M., Mant, A., Karnauchov, I., Brink, S., Herrmann, R.G., Klösgen, R.B. and Robinson, C. (1995) EMBO J. 14, 2715–2722.
[42] Chen, C. and Cheniae, G.M. (1996) in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. II, 329–332, Kluwer, Dordrecht.
[43] Chitnis, P.R., Purvis, D. and Nelson, N. (1991) J. Biol. Chem. 266, 20146–20151.
[44] Chu, H.-A., Nguyen, A.P. and Debus, R.J. (1994) Biochemistry 33, 6137–6149.
[45] Chu, H.-A., Nguyen, A.P. and Debus, R.J. (1994) Biochemistry 33, 6150–6157.
[46] Clausmeyer, S., Klšsgen, R.B. and Herrmann, R.G. (1993) J. Biol. Chem. 268, 13869–13876.
[47] Cline, K., Ettinger, W.F. and Theg, S.M. (1992) J. Biol. Chem. 267, 2688–2696.
[48] Cline, K., Henry, R., Li, C. and Yuan, J. (1993) EMBO J. 12, 4105–4114.
[49] Coleman, W.J. and Govindjee (1987) Photosyn. Res. 13, 199–223.
[50] Creighton, A.M., Hulford, A., Mant, A., Robison, D. and Robinson, C. (1995) 270, 1663–1669.
[51] Critchley, C., Andersson, B., Ryrie, I.J. and Anderson, J.M. (1984) Biochim. Biophys. Acta 767, 532–539.
[52] DeBoer, A.D. and Weisbeek, P.J. (1991) Biochim. Biophys. Acta 1071, 221–253.
[53] De Vitry, C., Olive, J., Drapier, D., Recouvreur, M. and Wollman, F.A. (1989) J. Cell Biol. 109, 991–1006.
[54] Debus, R.J. (1992) Biochim. Biophys. Acta 1102, 269–352.
[55] Durrant, J.R., Klug, D.R., Kwa, S.L.S., Van Grondelle, R., Porter, G. and Dekker, J.P. (1995) Proc. Natl. Acad. Sci. USA 92, 4798–4802.
[56] Eaton-Rye, J.J. and Murata, N. (1989) Biochim. Biophys. Acta 977, 219–226.
[57] Eaton-Rye, J.J. and Vermaas, W.F.J. (1991) Plant Mol. Biol. 17, 1165–1177.
[58] Enami, I., Kamino, K., Shen, J.R., Satoh, K. and Katoh, S. (1989) Biochim. Biophys. Acta 977, 33–39.
[59] Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sonoike, K., Inoue, Y. and Katoh, S. (1991) Biochim. Biophys. Acta 1060, 224–232.
[60] Enami, I., Mochizuki, Y., Takahashi, S., Kakuno, T., Horio, T., Satoh, K. and Katoh, S. (1990) Plant Cell Physiol. 31, 725–729.
[61] Enami, I., Murayama, H., Ohta, H., Kamo, M., Katsuyoshi, N. and Shen, J.-R. (1995) Biochim. Biophys. Acta 1232, 208–216.
[62] Enami, I., Ohta, S., Mitsuhashi, S., Takahashi, S., Ikeuchi, M. and Katoh, S. (1992) Plant Cell Physiol. 33, 291–297.
[63] Engels, D.H., Lott, A., Schmid, G.H. and Pistorius, E.K. (1994) Photosyn. Res. 42, 227–244.
[64] Ettinger, W.F. and Theg, S.M. (1992) Plant Physiol. 99, 791–793.
[65] Ford, R.C., Rosenberg, M.F., Shepherd, F.H., McPhie, P. and Holzenburg, A. (1995) Micron 26, 133–140.
[66] Frankl, L.K. and Bricker, T.M. (1992) Biochemistry 31, 11059–11064.
[67] Funk, C., Schröder, W.P., Napiwotzki, A., Tjus, S.E., Renger, G. and Andersson, B. (1995) Biochemistry 34, 11133–11141.
[68] Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130.
[69] Ghanotakis, D.F., Demetriou, D.M. and Yocum, C.F. (1987) Biochim. Biophys. Acta 891, 15–21.
[70] Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173.
[71] Ghanotakis, D.F., Topper, J.N. and Yocum, C.F. (1984) Biochim. Biophys. Acta 767, 524–531.
[72] Gilchrist, L.M. Jr., Ball, J.A., Randall, D.W. and Britt, R.D. (1995) Proc. Natl. Acad. Sci. USA 92, 9545–9549.
[73] Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Inoue, Y., Vermaas, W.F.J., Renger, G. (1994) Biochemistry 33, 12063–12071.
[74] Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Seeliger, A.G., Inoue, Y., Vermaas, W.F.J. and Renger, G. (1995) Biochemistry 34, 6847–6856.
[75] Görlach, J., Schmid, J. and Amrhein, N. (1993) Plant Cell Physiol. 34, 497–501.
[76] Gounaris, K., Chapman, D.J. and Barber, J. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 1, pp. 327–330, Kluwer, Dordrecht.
[77] Gray, J.C. and Row, P.E. (1995) Trends Cell Biol. 5, 243–247.
[78] Haag, E., Eaton-Rye, J.J., Renger, G. and Vermaas, W.F.J. (1993) Biochemistry 32, 4444–4454.
[79] Haag, E., Irrgang, K.-D., Boekema, E.J. and Renger, G. (1990) Eur. J. Biochem. 189, 47–53.
[80] Haehnel, W., Jansen, T., Gause, K., Klösgen, R.B., Stahl, B., Michl, D., Huvermann, B., Karas, M. and Herrmann, R.G. (1994) EMBO J. 13, 1028–1038.
[81] Halpin, C., Elderfield, P.D., James, H.E., Zimmermann, R., Dunbar, B. and Robinson, C. (1989) EMBO J. 8, 3917–3921.
[82] Han, K.-C. and Katoh, S. (1993) Plant Cell Physiol. 34, 585–593.
[83] Han, K.-C., Shen, J.-R., Ikeuchi, M. and Inoue, Y. (1994) FEBS Lett. 355, 121–124.
[84] Henry, R., Kapazoglou, A., McCaffery, M., Cline, K. (1994) J. Biol. Chem. 269, 10189–10192.
[85] Hind, G., Marshak, D.R., Coughlan, S.J. (1995) Biochemistry 34, 8157–8164.
[86] Ho, K.K., Ulrich, E.L., Krogmann, D.W. and Gomez-Lojero, C. (1979) Biochim. Biophys. Acta 545, 236–248.
[87] Hoganson, C.W., Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1989) Photosyn. Res. 22, 285–293.
[88] Hoganson, C.W., Lagenfeld, G., Andréasson, L.-E. and

Vänngard, T. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. III, pp. 319–322, Kluwer, Dordrecht.
[89] Holton, R.W. and Meyers, J. (1963) Science 142, 234–235.
[90] Holzenburg, A., Shepherd, F.H. and Ford, R.C. (1994) Micron 25, 447–451.
[91] Homann, P.H. (1988) Photosyn. Res. 15, 205–220.
[92] Homann, P.H. and Madabusi, L.M. (1993) Photosyn. Res. 35, 29–39.
[93] Hulford, A., Hazell, L., Mould, R. and Robinson, C. (1994) J. Biol. Chem. 269, 3251–3256.
[94] Ikeuchi, M., Shen, J.-R., Vermaas, W.F.J., Pakrasi, H.B., Diner, B.A., Nixon, P.J., Kurijama, A., Hirano, A. and Inoue, Y. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. II, pp. 175–178, Kluwer, Dordrecht.
[95] Irrgang, K.-D., Geiken, B., Lange, B. and Renger, G. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. II, pp. 417–420, Kluwer, Dordrecht.
[96] Irrgang, K.-D., Renger, G. and Vater, J. (1991) Eur. J. Biochem. 201, 515–522.
[97] Isogai, Y. Nishimura, M. and Itoh, S. (1987) Plant. Cell Physiol. 28, 1493–1499.
[98] Isogai, Y., Yamamoto, Y. and Nishimura, M. (1985) FEBS Lett. 187, 240–244.
[99] Jansen, T., Rother, C., Steppuhn, J., Reinke, H., Beyreuther, K., Jansson, C., Andersson, B. and Herrmann, R.G. (1987) FEBS Lett. 216, 234–240.
[100] Jansson, C., Åkerlund, H.-E. and Andersson, B. (1983) Photosyn. Res. 4, 271–279.
[101] Johnsson, G.N., Boussac, A. and Rutherford, A.W. (1994) Biochim. Biophys. Acta 1184, 85–92.
[102] Kang, C., Chitnis, P.R., Smith, S. and Krogmann, D.W. (1994) FEBS Lett. 344, 5–9.
[103] Kapazoglou, A., Sagliocco, F. and Dure III, L. (1995) J. Biol. Chem. 270, 12197–12202.
[104] Kashino, Y., Satoh, K. and Katoh, S. (1986) FEBS Lett. 205, 150–154.
[105] Kavelaki, K. and Ghanotakis, D.F. (1991) Photosyn. Res. 29, 149–155.
[106] Keegstra, K., Olsen, L.J. and Theg, S.M. (1989) Ann. Rev. Physiol. Plant. Mol. Biol. 40, 471–503.
[107] Klösgen, R.B., Brock, I.W., Herrmann, R.G. and Robinson, C. (1992) Plant Mol. Biol. 18, 1931–1934.
[108] Knoetzel, J. and Simpson, D.J. (1993) Plant Mol. Biol. 22, 337–345.
[109] Knott, T.G. and Robinson, C. (1994) J. Biol. Chem. 269, 7843–7846.
[110] Ko, K., Granell, A., Bennet, J. and Cashmore, A.R. (1990) Plant Mol. Biol. 14, 217–227.
[111] Koike, H. and Inoue, Y. (1985) Biochim. Biophys. Acta 807, 64–73.
[112] Konishi, T., Maruta, Y., Shinohara, K. and Watanabe, A. (1993) Plant Cell Physiol. 34, 1081–1087.
[113] Krinner, M., Hauska, G., Hurt, E. and Lockau, W. (1982) Biochim. Biophys. Acta 681, 110–117.
[114] Kuwabara, T. and Murata, N. (1979) Biochim. Biophys. Acta 581, 228–236.
[115] Kuwabara, T. and Murata, N. (1982) Biochim. Biophys. Acta 680, 210–215.
[116] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[117] Kuwabara, T. and Murata, N. (1983) Plant Cell Physiol. 24, 741–747.
[118] Kuwabara, T., Murata, T., Miyao, M. and Murata, N. (1986) Biochim. Biophys. Acta 850, 146–155.
[119] Kuwabara, T., Nagata, R. and Shinohara, K. (1989) Eur. J. Biochem. 186, 227–232.
[120] Kuwabara, T. Reddy, K.J. and Sherman, L.A. (1987) Proc. Natl. Acad. Sci. USA 84, 8230–8234.
[121] Kuwabara, T. and Suzuki, K. (1994) Plant Cell Physiol. 35, 665–675.
[122] Kuwabara, T. and Suzuki, K. (1995) Plant Cell Physiol. 36, 495–504.
[123] Kuwabara, T., Takeuchi, M., Honda, S., Nakajima, N., Watanabe, A. and Kondo, N. (1995) Plant Cell Physiol. 36, 435–439.
[124] Laider, V., Chaddock, A.M., Knott, T.G., Walker, D. and Robinson, C. (1995) J. Biol. Chem. 30, 17664–17667.
[125] Laudenbach, D.E., Erhardt, E., Green, L. and Grossman, A. (1991) J. Bacteriol. 173, 2751–2760.
[126] Laudenbach, D.E., Herbert, S.K., McDowell, C., Fork, D.C., Grossmann, A.R. and Strauss, N.A. (1990) Plant Cell 2, 913–924.
[127] Leuschner, C. and Bricker, T.M. (1996) Biochemistry 35, 4551–4557.
[128] Li, X., Henry, R., Yuan, J., Cline, K. and Hoffman, N.E. (1995) Proc. Natl. Acad. Sci. USA 92, 3789–3793.
[129] Ljungberg, U., Jansson, C., Andersson, B., Åkerlund, H.-E. (1983) Biochem. Biophys. Res. Commun. 113, 738–744.
[130] Ljungberg, U., Åkerlund, H.-E., Larsson, C. and Anderson, B. (1984) Biochim. Biophys. Acta 767, 145–152.
[131] Ljungberg, U., Åkerlund, H.-E. and Andersson, B. (1986) Eur. J. Biochem. 158, 477–482.
[132] MacDonald, G.M., Boerner, R.J., Everly, R.M., Cramer, A.W., Debus, R.J. and Barry, B.A. (1994) Biochemistry 33, 4393–4400.
[133] Mackle, M.M. and Zilinskas, B.A. (1994) J. Bacteriol. 176, 1857–1864.
[134] Mayes, S.R., Cook, K.M., Self, S.J., Zhang, Z. and Barber, J. (1991) Biochim. Biophys. Acta 1060, 1–12.
[135] Mayfield, S.P. (1991) Planta 185, 105–110.
[136] Mayfield, S.P., Bennoun, P. and Rochaix, J.-D. (1987) EMBO J. 6, 313–318.
[137] Mayfield, S.P., Rahire, M., Frank, G., Zuber, H. and Rochaix, J.-D. (1987) Proc. Natl. Acad. Sci. USA 84, 749–753.
[138] Mayfield, S.P., Rahire, M., Frank, G., Zuber, H. and Rochaix, J.-D. (1989) Plant Mol. Biol. 12, 683–693.
[139] Meadows, J.W., Holford, A., Raines, C.A. and Robinson, C. (1991) Plant Mol. Biol. 16, 1085–1087.
[140] Mei, R., Green, J.P., Sayre, R.T. and Frasch, W.D. (1989) Biochemistry 28, 5560–5567.
[141] Mei, R. and Yocum, C.F. (1992) Biochemistry 31, 8449–8454.

[142] Merrit, S., Ernfors, P., Ghanotakis, D.F. and Yocum, C.F. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 689–692, Martinius Nijhoff Publishers, The Netherlands.
[143] Michel H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[144] Michl, D., Robinson, C., Shackleton, J.B., Herrmann, R.G. and Klösgen, R.B. (1994) EMBO J. 13, 1310–1317.
[145] Miller, A.-F., De Paula, J.C. and Brudwig, G.W. (1987) Photosyn. Res. 12, 205–218.
[146] Mishra, R.K. and Ghanotakis, D.F. (1993) Photosyn. Res. 36, 11–16.
[147] Mishra, R.K. and Ghanotakis, D.F. (1994) Photosyn. Res. 42, 37–42.
[148] Miura, K., Shimazu, T., Motoki, A., Kanai, S., Hirano, M. and Katoh, S. (1993) Biochim. Biophys. Acta 1172, 357–360.
[149] Miyao, M., Fujimura, Y. and Murata, N. (1988) Biochim. Biophys. Acta 936, 465–474.
[150] Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93.
[151] Miyao, M. and Murata, N. (1983) FEBS Lett. 164, 375–378.
[152] Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120.
[153] Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354.
[154] Miyao, M. and Murata, N. (1986) Photosyn. Res. 10, 489–496.
[155] Miyao, M. and Murata, N. (1989) Biochim. Biophys. Acta 977, 315–321.
[156] Miyao, M., Murata, M., Lavorel, J., Maison-Peteri, B., Boussac, A. and Etienne, A.-L. (1987) Biochim. Biophys. Acta 890, 151–159.
[157] Miyao-Tokutomi, M. and Inoue, Y. (1992) Biochemistry 31, 526–532.
[158] Mizobuchi, A. and Yamamoto, Y. (1989) Biochim. Biophys. Acta 977, 26–32.
[159] Murata, N. and Miyao, M. (1985) Trends Biochem. Sci. 10, 122–124.
[160] Murata, N., Miyao, M., Omata, T., Matsunami, H. and Kuwabara, T. (1984) Biochim. Biophys. Acta 765, 363–369.
[161] Nakai, M., Goto, A., Nohara, T., Sugati, D. and Endo, T. (1994) J. Biol. Chem. 269, 31338–31341.
[162] Nakai, M., Sugita, D., Omata, T. and Endo, T. (1993) Biochim. Biophys. Res. Commun. 193, 228–234.
[163] Nakatani, H.Y. (1984) Biochem. Biophys. Res. Commun. 120, 299–304.
[164] Nilsson, F., Andersson, B. and Jansson, C. (1990) Plant Mol. Biol. 14, 1051–1054.
[165] Nishiyama, Y., Hayashi, H., Watanabe, T. and Murata, N. (1994) Plant Physiol. 105, 1313–1319.
[166] Nohara, T., Nakai, M., Goto, A. and Endo, T. (1995) FEBS Lett. 364, 305–308.
[167] Odom, W.R. and Bricker, T.M. (1992) Biochemistry 31, 5616–5620.
[168] Oh-oka, S., Tanaka, K., Wada, K., Kuwabara, T. and Murata, N. (1986) FEBS Lett. 197, 63–66.
[169] Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260.
[170] Ono, T. and Inoue, Y. (1984) FEBS Lett. 166, 381–384.
[171] Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–184.
[172] Ono, T. and Inoue, Y. (1985) Biochim. Biophys. Acta 806, 331–340.
[173] Ono, T. and Inoue, Y. (1986) Biochim. Biophys. Acta 850, 380–389.
[174] Ono, T. and Inoue, Y. (1988) FEBS Lett. 227, 147–152.
[175] Ono, T. and Inoue, Y. (1990) Biochim. Biophys. Acta 1020, 269–277.
[176] Ono, T., Izawa, S. and Inoue, Y. (1992) Biochemistry 31, 7648–7655.
[177] Palomares, R., Herrmann, R.G. and Oelmüller, R. (1993) Planta 190, 305–312.
[178] Pauly, S., Schlodder, E. and Witt, H.T. (1992) Biochim. Biophys. Acta 1099, 203–210.
[179] Phibrick, J.B. and Zilinkas, B.A. (1988) Mol. Gen. Genet. 212, 418–425.
[180] Philbrick, J.B., Diner, B.A. and Zilinskas, B.A. (1991) J. Biol. Chem. 266, 13370–13376.
[181] Renger, G. (1993) Photosyn. Res. 38, 229–247.
[182] Robinson, C., Cai, D., Hulford, A., Brock, I.W., Michl, D., Hazell, L., Schmidt, I., Herrmann, R.G. and Klösgen, R.B. (1994) EMBO J. 13, 179–285.
[183] Robinson, C. and Klösgen, R.B. (1994) Plant Mol. Biol. 26, 15–24.
[184] Rolfe, S.A. and Bendall, D.S. (1989) Biochim. Biophys. Acta 973, 220–226.
[185] Rother, C., Jansen, T., Tyagi, A., Tittgen, J. and Herrmann, R.G. (1986) Curr. Genet. 11, 171–176.
[186] Rova, M., Franzén, L.-G., Fredriksson, P.-O. and Styring, S. (1994) Photosyn. Res. 39, 75–83.
[187] Rutherford, A.W. (1989) Trends Biochem. Sci. 14, 227–232.
[188] Rutherford, A.W., Zimmermann, J.-L. and Boussac, A.W. (1992) in The Photosystems: Structure, Function and Molecular Biology (Barber, J., ed.), pp. 179–229, Elsevier, Amsterdam.
[189] Ryrie, I.J., Young, S. and Andersson, B. (1984) FEBS Lett. 177, 269–273.
[190] Santini, C., Valentino, T., Tognon, G., Ghiretti Magaldi, A. and Bassi, R. (1994) Eur. J. Biochem. 221, 307–315.
[191] Sayre, R.T., Andersson, B. and Bogorad, L. (1986) Cell 47, 601–608.
[192] Seidler, A. (1994) Biochim. Biophys. Acta 1187, 73–79.
[193] Seidler, A. (1994) Protein Engineering 7, 1277–1280.
[194] Seidler, A. and Michel, H. (1990) EMBO J. 9, 1743–1748.
[195] Seidler, A. and Michel, H. (1991) in Trends in Photosynthesis Research (Barber, J., Medrano, M. and Guerrero, M.G., eds.) pp. 149–156, Intercept, Andover.
[196] Seidler, A., Röll, K. and Michel, H. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. 2, pp. 409–412, Kluwer, Dordrecht.
[197] Seidler, A. and Rutherford, A.W. (1996) Biochemistry, in press.

[198] Seidler, A., Rutherford, A.W. and Michel, H. (1996) in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. II, pp. 259–263, Kluwer, Dordrecht.
[199] Seidler, A., Rutherford, A.W. and Michel, H. (1996) Plant Mol. Biol. 31, 183–188.
[200] Shen, J.-R., Burnap, R.L. and Inoue, Y. (1995) Biochemistry 34, 12661–12668.
[201] Shen, J.-R. and Katoh, S. (1991) Plant Cell. Physiol. 32, 439–446.
[202] Shen, J.-R., Ikeuchi, M. and Inoue, Y. (1992) FEBS Lett. 301, 145–149.
[203] Shen, J.-R., and Inoue, Y. (1993) Biochemistry 32, 1825–1832.
[204] Shen, J.-R., and Inoue, Y. (1993) J. Biol. Chem. 268, 20408–20413.
[205] Shen, J.-R., Vermaas, W.F.J. and Inoue, Y. (1995) J. Biol. Chem. 270, 6901–6907.
[206] Shigemori, Y., Inagaki, J., Mori, H., Nishimura, M., Takahashi, S. and Yamamoto, Y. (1994) Plant Mol. Biol. 24, 209–215.
[207] Shukla, V.K., Anbudurai, P.R., Wu, R. and Pakrasi, H.B. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. III, pp. 409–412, Kluwer, Dordrecht.
[208] Stader, J., Benson, S.A. and Silhavy, T.J. (1986) J. Biol. Chem. 261, 15075–15080.
[209] Stewart, A.C., Ljungberg, U., Akerlund, H.-E. and Andersson, B. (1985) Biochim. Biophys. Acta 808, 355–362.
[210] Styring, S., Miyao, M. and Rutherford, A.W. (1987) Biochim. Biophys. Acta 767, 32–38.
[211] Tae, G.-S. and Cramer, W.A. (1989) FEBS Lett. 259, 161–164.
[212] Tae, G.-S. and Cramer, W.A. (1992) Biochemistry 31, 4066–4074.
[213] Tamura, N. and Cheniae, G.M. (1985) Biochim. Biophys. Acta 809, 245–259.
[214] Tamura, N. and Cheniae, G.M. (1986) FEBS Lett. 200, 231–236.
[215] Tamura, N. and Cheniae, G.M. (1987) Biochim. Biophys. Acta 890, 179–194.
[216] Tamura, N., Inoue, H. and Inoue, Y. (1990) Plant Cell Physiol. 31, 469–477.
[217] Tanaka, S. and Wada, K. (1988) Photosyn. Res. 17, 255–266.
[218] Tang, X.S. and Diner, B.A. (1994) Biochemistry 33, 4594–4603.
[219] Tyagi, A., Hermans, J., Steppuhn, J., Jansson, C., Vater, F. and Herrmann, R.G. (1987) Mol. Gen. Genet. 207, 288–293.
[220] Van Mieghem, F.J.E., Satoh, K. and Rutherford, A.W. (1991) Biochim. Biophys. Acta 1058, 379–385.
[221] Van Spanje, M., Dirkse, W.G., Nap, J.-P. and Stiekema, W.J. (1991) Plant Mol. Biol. 17, 157–160.
[222] Van Vliet, P., Boussac, A. and Rutherford, A.W. (1994) Biochemistry 33, 12998–13004.
[223] Vass, I., Cool, K.M., Deàk, Z., Mayes, S.R., Barber, J. (1992) Biochim. Biophys. Acta 1102, 195–201.
[224] Vass, I., Ono, T. and Inoue, Y. (1987) Biochim. Biophys. Acta 892, 224–235.
[225] Vermaas, W.F.J., Styring, S., Schröder, W.P. and Andersson, B. (1993) Photosyn. Res. 38, 249–263.
[226] Völker, M., Ono, T., Inoue, Y. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34.
[227] Völker, R. and Barkan, A. (1995) EMBO J. 14, 3905–3914.
[228] von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989) Eur. J. Biochem. 180, 535–545.
[229] Wales, R., Newman, B.J., Pappin, D. and Gray, J.C. (1989) Plant Mol. Biol. 12, 439–451.
[230] Watanabe, A., Minami, E., Murase, M., Shinohara, K., Kuwabara, T. and Murata, N. (1987) in Progress in Photosynthesis Research (Biggens, L., ed.), Vol. IV, pp. 629–636, Kluwer, Dordrecht.
[231] Webber, A.N. and Gray, J.C. (1989) FEBS Lett. 249, 79–82.
[232] Wedel, N., Klein, R., Ljungberg, U., Andersson, B. and Herrmann, R.G. (1992) FEBS Lett. 314, 61–66.
[233] Wenng, A., Ehmann, B. and Schäfer, E. (1989) FEBS Lett. 246, 140–144.
[234] Westhoff, P., Schrubar, H., Oswald, A., Streubel, M. and Offermann, K. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. III, pp. 483–490, Kluwer, Dordrecht.
[235] Xu, Q. and Bricker, T.M. (1992) J. Biol. Chem. 267, 25816–25821.
[236] Xu, Q., Nelson, J. and Bricker, T.M. (1994) Biochim. Biophys. Acta 1188, 427–431.
[237] Yachandra, V., DeRose, V.J., Latimer, M.J., Mukerji, I., Sauer, K. and Klein, M.P. (1993) Science 260, 675–679.
[238] Yamamoto, Y. (1988) J. Biol. Chem. 263, 497–500.
[239] Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268.
[240] Yamamoto, Y. and Kubota, F. (1987) Biochim. Biophys. Acta 893, 579–583.
[241] Yamamoto, Y., Nakayama, S., Cohn, C.L. and Krogmann, D.W. (1987) Arch. Biochem. Biophys. 255, 156–161.
[242] Yamamoto, Y., Shimada, S. and Nishimura, M. (1983) FEBS Lett. 151, 49–53.
[243] Yamamoto, Y., Shinkai, H., Isogai, Y., Matsuura, K. and Nishimura, M. (1984) FEBS Lett. 175, 429–432.
[244] Yocum, C.F. (1991) Biochim. Biophys. Acta 1059, 1–15.
[245] Yuan, J., Henry, R., McCaffery, M. and Cline, K. (1993) Science 266, 796–798.
[246] Zimmermann, J.-L., Boussac, A. and Rutherford, A.W. (1993) Biochemistry 32, 4831–4841.
[247] Zhang, L., Pakrasi, H.B. and Whitmarsh, J. (1994) J. Biol. Chem. 269, 5063–5042.